(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 March 2005 (24.03.2005)

PCT

(10) International Publication Number
WO 2005/026389 A2

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number:
PCT/EP2004/009546

(22) International Filing Date: 26 August 2004 (26.08.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
03 01 9521.8 29 August 2003 (29.08.2003) EP

(71) Applicant *(for all designated States except US)*: **MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN e.V.** [DE/DE]; Berlin (DE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **SOLDATOV, Aleksey** [RU/DE]; Schönhauserstrasse 16, 12169 Berlin (DE). **BORODINA, Tatiana** [RU/DE]; Schönhauserstrasse 16, 12169 Berlin (DE). **LEHRACH, Hans** [AT/DE]; Terrassenstr. 31, 14129 Berlin (DE).

(74) Agent: **VOSSIUS & PARTNER**; Siebertstrasse 4, 81675 Munich (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: LIGATION-BASED METHOD OF ANALYSIS OF SINGLE NUCLEOTIDE POLYMORPHISMS ON GENOMIC DNA

(57) Abstract: The present invention relates to a method for the detection of the allelic state of n single nucleotide polymorphism (SNP)-loci on genomic DNA comprising contacting said genomic DNA containing SNP-loci with n detector oligonucleotide sets (DO-sets).

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Ligation-based method of analysis of single nucleotide polymorphisms on genomic DNA.

The present invention relates to a method for the detection of the allelic state of n single nucleotide polymorphism (SNP)-loci on genomic DNA comprising the steps of (a) contacting said genomic DNA containing SNP-loci with n detector oligonucleotide sets (DO-sets), wherein n represents the number of specific loci to be tested, and wherein each DO-set represents a specific collection of (aa) a first detector oligonucleotide (DO), wherein said first DO comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination; and a Region III, for locus specific hybridisation, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and (ab) a second DO, wherein said second DO comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region

III′ is specific for said first allelic state of said SNP-locus; and (ac) a third DO, wherein said third DO is 5′-phosphorilated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridisation, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ terminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets; (b) carrying out a cyclic ligation reaction simultaneously with n DO-sets, wherein for each particular DO set of step (a) depending on the allelic state of the corresponding locus, either the first DO or the second DO or both DOs are linked to the third DO; (c) carrying out a first PCR-amplification reaction on the ligated products with a common first and a common second PCR primer, wherein said common first PCR primer corresponds in sequence to Region I/I′ of the first and second detector oligonucleotide of step (a) and said common second PCR primer is complementary in sequence to Region V of the third DO of step (a); (d) separating the amplification products into n aliquots; (e) carrying out for each aliquot (ea) a second PCR-amplification reaction with the common first PCR primer of step (c) and a second PCR primer which is either complementary to Region IV or, optionally, to Region VI of the third DO of step (a), simultaneously or step-by-step with (eb) an allele discrimination detection reaction which detects Regions II and II′ in the amplification products.

Furthermore, the invention relates to the detector oligonucleotides and to a kit comprising the detector oligonucleotides of the invention

In this specification, a number of documents are cited. The disclosure content of these documents including manufacturers′ manuals is herewith incorporated by reference in its entirety.

According to the formal definition, a Single Nucleotide Polymorphism (SNP) represents a DNA sequence variant of a single base pair, with the minor allele occurring in more than 1% of a given population. In principal, SNPs may be four-allelic, because any nucleotide position may exist in four allelic variants (the number of different bases in genomic DNA). However, the overwhelming majority of SNPs is biallelic, therefore the general task of SNP detection is often formulated as recognition of biallelic markers.

Compared to other genetic markers, SNPs cover genomes with a very high density. For example in the human genome SNPs have been estimated to occur approximately every thousand nucleotides (Li and Sadler 1991). In addition, SNPs have a very low mutation rate per generation (10^{-8}) and thus may serve for studying of molecular evolution (Crow 1995; Li at al. 1996). SNP markers may be used for person identification in criminalistics and for selection of individual therapy in medicine and pharmaceutics. Besides, SNP genotyping is extremely valuable for the identification of genes responsible for particular traits and disorders in medicine and agriculture (Brookes 1999).

The broad field of applications of SNPs induced a great interest and a pressing need for effective instruments of SNP discovery (search for new SNPs) and detection (recognition of already known SNPs). Nowadays, the limiting factor for using of SNP genotyping in medicine, science and industry is the efficiency of the existing technologies (reliability, price and throughput). Existing approaches are adequate for analysis either of several loci in a large number of samples: TaqMan (Holland et al. 1991; Livak et al. 1995), Invader (Lyamichev and Neri 2003), MALDI-based methods (Tost and Gut 2002), Amplifluor (Myakishev et al. 2001; Hawkins et al. 2002), DASH (Howell et al. 1999; Prince et al. 2001), SNaPshot (Applied Biosciences, PE Corporation); or a large number of loci in few samples: microarray-based (Affymetrix, Inc.; Wang et al. 1998) or padlock-based (Hardenbol eal. 2003) genotyping. However, many applications, especially the association studies of polygenic traits require the scoring of hundreds of thousands of SNP in thousands of individuals. Such studies would cost in the range of 10^7 $ and are too expensive to be routine

now. Significant improvement of the available techniques or development of new technologies is required not only to make the present research cheaper, but also to open previously inaccessible areas. The existing methods have the following disadvantages that restrict their application:

(i) necessity to adjust conditions of individual SNP-genotyping reactions (TaqMan, Invader, DASH);

(ii) requirement for the preliminary amplification of SNP-containing regions of genomic DNA (MALDI-based methods, DASH, SnaPshot, microarray-based methods);

(iii) high set-up price (TaqMan, microarray-based and padlock-based methods);

(iv) complex experimental procedure which is difficult to automate (SnaPshot, microarray-based and padlock-based methods);

(v) requirement for very expensive equipment (MALDI-based methods).

As discussed above, the methods of the prior art for analysis of single nucleotide polymorphisms manifest substantial disadvantages. Thus, there was a need for means that overcome said disadvantages. The technical problem underlying the present invention was therefore to provide such means.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims. Accordingly, the present invention relates to a method for the detection of the allelic state of n single nucleotide polymorphism (SNP)-loci on genomic DNA comprising the steps of (a) contacting said genomic DNA containing SNP-loci with n detector oligonucleotide sets (DO-sets), wherein n represents the number of specific loci to be tested, and wherein each DO-set represents a specific collection of (aa) a first detector oligonucleotide (DO), wherein said first DO comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination; and a Region III, for locus specific hybridisation, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and (ab) a

second DO, wherein said second DO comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and (ac) a third DO, wherein said third DO is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridisation, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ terminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets; (b) carrying out a cyclic ligation reaction simultaneously with n DO-sets, wherein for each particular DO set of step (a) depending on the allelic state of the corresponding locus, either the first DO or the second DO or both DOs are linked to the third DO; (c) carrying out a first PCR-amplification reaction on the ligated products with a common first and a common second PCR primer, wherein said common first PCR primer corresponds in sequence to Region I/I′ of the first and second detector oligonucleotide of step (a) and said common second PCR primer is complementary in sequence to Region V of the third DO of step (a); (d) separating the amplification products into n aliquots; (e)

carrying out for each aliquot (ea) a second PCR-amplification reaction with the common first PCR primer of step (c) and a second PCR primer which is either complementary to Region IV or, optionally, to Region VI of the third DO of step (a), simultaneously or step-by-step with (eb) an allele discrimination detection reaction which detects Regions II and II′ in the amplification products.

The term "single nucleotide polymorphism (SNP)" is used in accordance with the present invention in a looser sense, than in the formal definition: "SNP represents a natural substitution of one DNA base to another, the least common allele occurring in more than 1% of a given population". In the present invention SNPs are referred to as being short allelic variants, i.e. substitutions or small insertions-deletions, wherein minimum allele frequencies for the polymorphisms are not taken into consideration. Such loose definition is widely used. For example, the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/) also uses said SNP definition, which does not take into consideration allelic frequencies.

The term "locus specific hybridisation" as used in connection with the present invention means specific hybridisation of the detector oligonucleotides with the definite SNP-containing region in the genomic DNA.

The term "allelic state" used in accordance with the present invention means one of several existing variants of the nucleotide sequence of the particular DNA region.

The term "allele discrimination" refers to the recognition of the allelic state of the particular SNP locus.

In accordance with the present invention, the term "cyclic ligation reaction" refers to the ligation reaction performed with thermostable DNA ligase where the temperature of the reaction is periodically increased above the melting temperature of the DNA-substrate.

The present invention relates to a high throughput method of SNP genotyping, particularly suitable for the analysis of a large number of SNP's (some hundreds) in a large number of samples (thousands). The method of the invention is based on a

ligation detection reaction (LDR) performed directly on genomic DNA. During ligation the biallelic state of the SNP locus is converted into a bimarker state of ligated detector oligonucleotide. The detector oligonucleotides of the present invention have a special design, which allows amplifying them and determining the state of the allelic marker. The procedure

(i) uses a standard protocol for any SNP locus;
(ii) is performed directly on the genomic DNA without preliminary PCR amplification;
(iii) has an acceptable set-up price;
(iv) includes a few simple pipeting steps;
(v) requires common laboratory equipment.

Easy experimental procedure and the standard protocol for all SNP loci permits to perform the method of the invention on automated liquid-handling system.

The present invention relates to a new ligation detection reaction (LDR) -based method of SNP genotyping. The specific design of the detector oligonucleotides of the invention permits to carry out the method of the invention at high sensitivity, which means that it can be performed directly on genomic DNA. The method of the invention comprises only a minimum number of steps and is the same for different SNP loci, i.e. no locus-specific optimization is required. Several SNP loci may be analysed simultaneously on the same sample of genomic DNA.

The ligation detection reaction (LDR) is based on the faster ligation of matched compared to mismatched nicks (Figure 1). It is one of the most promising approaches for the analysis of SNP variants because it is more accurate than hybridisation methods and permits simultaneous analysis of several SNP loci in one reaction mixture. Some other LDR-based SNP genotyping methods which are sensitive enough to be performed on genomic DNA were described previously: Barany et al. 2001; Faruqi et al. 2001; P. Lizardi et al. 1998; Hardenbol 2003. Barany et al. (2001) describe an LDR-PCR protocol for parallel analysis of several SNP loci on genomic DNA. However, additional separation procedures such as electrophoresis or microarray hybridisation are required for analysis of LDR-PCR reaction. The method of Faruqi et al. (2001) uses activated fluorescent labels (Amplifluor primers) and permits to detect the result of genotyping reaction in closed tubes. This method,

however, cannot be used for simultaneous analysis of several SNPs, and involves expensive padlock probes. Hardenbol et al. (2003) managed to perform simultaneous padlock-based genotypings of about 1000 loci. This procedure, however, also exhibits substantial disadvantages. The use in Hardenbol et al. of padlock probes for ligation detection excludes the possibility of cyclic ligation - at least, without preliminary fragmentation of the genomic DNA - and thus restricts the sensitivity of the procedure. Furthermore, gap filling reaction are performed in separate tubes which, together with the use of several microarrays for one genotyping reaction decreases the reproducibility of the assay. Additionally, the detection procedure in Hardenbol et al. is very complex, includes several enzymatic steps, and is problematic for automation.

The general design of the detector oligonucleotides of the present invention is shown in Figure 2. Three DOs are used for each biallelic locus. These DOs have distinct regions for (i) amplification, (ii) allele and (iii) locus discrimination. Such design requires relatively long oligonucleotides, but as will be shown below DOs have a distinct block structure and may be cost-efficiently produced by ligation-based synthesis (Borodina et al. 2003).

The design of detector oligonucleotides with separate regions may have some variations for different detection schemes. Features in the DOs, which are conserved in any case, are:

- Regions for amplification (I & V) which are constant for all DOs. They are located such that amplicons appear only as a result of ligation. If no ligation occurs, no amplicons are generated.
- Two different allele-discrimination regions (II and II'), which are used for biallelic loci. They are located in the same DOs as the allele-specific regions (III). If the locus is three- or four-allelic, then three or four allele-discrimination regions should be used.

The scheme of the genotyping reaction is shown in Figure 3. In step (1) ligation detection reaction with n DO sets is performed on the same sample of genomic DNA. Depending on the allelic state of SNP-loci particular allele-discrimination Regions (II or II') are included in ligated pairs of detector oligonucleotides: Region II for locus_01

and Region II' for locus_n. Thus, the biallelic state of SNP loci is converted into the bimarker state of ligated DOs. The first marker is Region II and the second marker is Region II' (shown black and white in Figure 3).

The marker state of each particular SNP locus is determined in separate amplification reactions with one common and one locus-specific PCR primer (step 3). Locus-specific primers (#PCR_01r, ... , #PCR_nr) are used to distinguish single amplicon from a complex mixture. An intermediate preamplification step 2 is introduced into the protocol to prevent decrease of sensitivity due to the division of the ligation mixture into a number of separate aliquots ("n" in Figure 3).

The use of the specialized locus-discrimination Region VI in the design of the DOs of the invention is advantageous, because the same "n" locus-specific PCR-primers are used in different "n-loci" DO-sets. This makes the second amplification reaction more reliable and reproducible. The only locus-specific components are then locus-specific regions which participate in the ligation reaction. However (as demonstrated in Example 2 below) the locus-specific Region IV may be used instead of Region VI for locus discrimination. This substitution is advantageous for SNP-detection projects with a low number of SNP-loci, because exclusion of Region VI leads to shortening of "right" DOs.

As was shown in Amplification Fragment Length Polymorphism (AFLP) – experiments the initial ratio of hundreds of amplicons is preserved when amplification is performed with common primers (Vos et al. 1995). The design of the DOs of the invention permits to use two common primers for the amplification of all ligated DOs on the step 2 and thus to keep the initial ratio of different amplicons during PCR. Also on the step 3 in each separate tube only two primers are used for amplification of both allelic variants.

When closed tube detection is performed in step 3 of Figure 3 (as in Example 2) the whole procedure involves only three pipeting steps: (i) addition of genomic DNA to the Ligation Premixture; (ii) addition of the First PCR Mix to the ligation reaction; (iii) aliquoting of the amplified material to the tubes with the Second PCR Mixtures. These simple operations may be performed by automated liquid-handling system.

The possibility of simultaneous analysis of a number of SNP loci on the same portion of genomic DNA makes the method of the invention an adequate basis for clinical assays where only restricted amount of biological material is available for the analysis.

In a preferred embodiment of the method of the present invention, the first PCR amplification reaction of step 1(c) is performed simultaneously or step by step with the allele discrimination reaction of step 1(eb) and steps 1(d) and 1(ea) are omitted.

With this preferred embodiment of the invention, it is possible to test only one single SNP locus. The scheme of the genotyping reaction for the detection of one single SNP locus is shown in Figure 4. This is a two-step reaction. First, depending on the allelic state of SNP-locus, a particular allele-discrimination Region (II or II') is included in ligated pairs of DOs: Region II in case of Allele 1 and Region II' in case of Allele 2. The marker status of the locus is then determined in the amplification reaction with common primers.

If closed tube detection is used on step 2 of Figure 4 (as in Example 1), the method of said preferred embodiment of the invention involves two pipeting steps: (i) addition of genomic DNA to the Ligation Premixture; (ii) addition of universal PCR Mix to the ligation reaction. Neither step (i) nor step (ii) operate with amplified DNA. Said method is therefore safe in terms of cross-contamination, and can be applied for clinical and criminalistic applications. Furthermore, it may be advantageous for genotyping projects involving analysis only of a few loci in large number of samples.

In another preferred embodiment of the method of the present invention a first oligonucleotide labelled with a first detectable label and able to hybridize with Region II and a second oligonucleotide labelled with a second detectable label and able to hybridize with Region II′ are used in the allele discrimination reaction of step 1 (eb).

In a more preferred embodiment of the method of the present invention said first and second detectable labels are two distinguishable fluorescent labels and their

fluorescence is suppressed by quenchers located in close vicinity to said fluorescent labels and the presence of Region II in the amplification products in the allele discrimination reaction of step 1 (eb) induces activation of the first fluorescent label but does not activate the second fluorescent label and the presence of Region II′ in the amplification products in the allele discrimination reaction of step 1 (eb) induces activation of the second fluorescent label, but does not activate the first fluorescent label.

This embodiment of the method of the present invention is carried out as a closed-tube detection assay. The term "closed tube detection" refers to the methods of analysis of reaction without contacting the reaction solution by some probe and without taking aliquots for examination. Commonly, such detection is performed by optical methods such as, for example, measurement of adsorbtion, fluorescence, fluorescence polarization.

In a further preferred embodiment of the method of the present invention both said quencher and said first fluorescent label are covalently attached to the first oligonucleotide and both said quencher and said second fluorescent label are covalently attached to the second oligonucleotide and spacial separation is performed by Taq polymerase 5′-exonuclease digestion of the first oligonucleotide hybridized to Region II or the second oligonucleotide hybridized to Region II′.

This preferred embodiment of the present invention makes use of the method of close-tube detection with fluorescent labels and 5′ exonuclease assay (TaqMan: Holland et al. 1991; Livak et al. 1995)

In another preferred embodiment of the method of the present invention both said quencher and said first fluorescent label are covalently attached to the first oligonucleotide and both said quencher and said second fluorescent label are covalently attached to the second oligonucleotide and each of the first and second oligonucleotide contains two short complementary regions and said first or second fluorescent label and said quencher are brought together when said complementary regions are hybridised with each other, and the separation of said first or second

fluorescent label from the quencher is due to change of spacial conformation of the first or second labelled oligonucleotide after hybridization with Region II or II′.

This preferred embodiment of the present invention makes use of the method of close-tube detection with fluorescent labels and Molecular Beacons (Tyagi et al. 1998).

In yet another preferred embodiment of the method of the present invention said first and second oligonucleotides contain covalently attached fluorescent labels and said quenchers are attached to additional oligonucleotides and said additional oligonucleotides are complementary to said first and second oligonucleotides and said first or second fluorescent label and said quencher are brought together when said additional oligonucleotides are hybridised with said first and second oligonucleotides and said quencher is separated from said fluorescent label when said first and second oligonucleotides hybridize with Regions II or II′.

This preferred embodiment of the present invention makes use of the method of close-tube detection with fluorescent labels and composite FRET primers (Li et al. 2002).

The above mentioned embodiments of the method of the present invention relate to different techniques of identification of the marker state and are all based on the Fluorescent Resonance Energy Transfer (FRET) principle. It is also possible to base closed-tube detection on other principles, for example on fluorescence polarisation detection (Chen et al. 1999).

In a preferred embodiment of the method of the present invention the allele discrimination reaction of step 1(eb) is performed with a microarray detection system simultaneously or step by step with the first amplification reaction of step 1(c), wherein individual elements of said microarray are able to hybridize with locus-specific Regions IV or, optionally, Regions VI of amplification products and Regions II and II' in the amplification products are detected by hybridization with the first and second oligonucleotides labeled with two distinguishable fluorescent labels.

This embodiment of the method of the present invention includes two basic steps: (i) the conversion of the state of the biallelic SNP loci into the state of a universal markers (Region II and II') and (ii) the identification of the marker state. These steps are independent from each other and may be optimized or redesigned separately. As already outlined above in other terms, the design of detector oligonucleotides may have some variations for different techniques of identification of the marker state. An example of microarray-based detection procedure with locus-discriminating Region VI located in the "left" DO is shown in Figure 5. The first two steps (multilocus ligation and PCR amplification with common primers) are the same as in Figure 3. Optional digestion by restriction endonuclease in step 3 removes any locus-specific sequences from amplicons. This prevents locus-specific variation of hybridisation velocity. Single-stranded hybridisation probes are prepared in this step by purification on streptavidine paramagnetic particles. Step 4 is hybridisation of single-stranded probes with microarray and with two (for biallelic loci) fluorescently labelled (Cy3 and Cy5 in Figure 5) allele-discrimination oligonucleotides complementary to Regions II and II', corresponding to the first and the second oligonucleotides of the present invention labelled with detectable labels. Each spot of the microarray contains DNA fragments hybridising with particular locus-discrimination Region VI. Locus-discrimination sequences are the same for different *n*-loci sets, so the same universal microarray is used for any genotyping. Simultaneous hybridisation of single-stranded amplicons with microarray and allele-discrimination oligonucleotides (sandwich-hybridisation) allows to distinguish both loci and allelic variants in a single hybridisation reaction. Loci are distinguished according to position on microarray. Allelic states of loci are distinguished according to fluorescent signals in corresponding spots (Cy3 and/or Cy5).

In another preferred embodiment of the method of the present invention said common second PCR primer is destroyed after the first PCR-amplification of step 1(c).

In a more preferred embodiment of the method of the present invention said common second PCR primer contains Uridine- or Ribo-nucleotides and is destroyed by Uracile DNA Glycosylase (UDG) or RNase included in said second PCR-amplification reaction of step 1(e).

The marker state of each particular SNP locus is determined in separate amplification reactions with one common and one locus-specific PCR primer (step 3 in Figure 3). Locus-specific primers (#PCR_01r, ... , #PCR_nr) are used to distinguish single amplicon from a complex mixture. However, the rest of common PCR primers from the First PCR amplification (step 2 in Figure 3) may decrease the specificity of the reaction. Uridine- or Ribo-nucleotides may be introduced into the sequence of interfering primer to have a possibility to destroy it before the Second PCR reaction. The use of Uracile DNA Glycosylase (UDG) or RNase is convenient, because these enzymes may be included into the Second PCR Mixtures without modifying the experimental procedure.

In a further preferred embodiment of the method of the present invention said Regions I and I′ have identical sequences.

In an additionally preferred embodiment of the method of the present invention said first, second and third DOs are prepared by ligation based synthesis.

Though left and right detector oligonucleotides may be prepared by conventional phosphoramidite technology, their block structure makes them appropriate for ligation-based synthesis (LBS) (Borodina et al. 2003). In LBS the constant parts of oligonucleotides have to be synthesized only once. "Left" DOs are combinations of allele specific Regions III with two (for biallelic SNPs) conservative presynthesized oligos (Regions I & II or I & II'). "Right" detector oligonucleotides are combinations of locus specific parts (Region IV) with n conservative presynthesized oligos (Region VI & V). For example, LBS of 100 sets of DOs (1.5nmol – scale synthesis of three DOs per each locus) costs about 5000$, i.e. 50$ per one DO-set. The price is therefore about six fold less compared to conventional phosphoramidite technology. Due to the low working concentration (1nM) a single 1.5nmol-scale synthesis provides enough DOs for about 300000 genotyping reactions.

An additional advantage of ligation-based synthesis is the possibility to include at low cost modified bases in the common part of composite oligonucleotides, for example exponuclease resistant groups. Such modification may be used to remove unligated

DOs by exonuclease treatment after ligation detection reaction. If exonuclease-resistant regions are included in 5'-terminal part of the "left" DO and in the 3'-terminal part of the "right" DO, then unligated DOs would have one exonuclease-sensitive end, but ligated DOs would be protected from both ends. An example of exonuclease-resistant group is phosphothioate-binding. When included in 3'-end they protect oligonucleotides from digestion by Exonuclease I, Exonuclease III (Lutz et al. 2001), and 3'->5' exonuclease activity of proofreading DNA polymerases (T4 DNA polymerase, Pfu polymerase) (Dean et al. 2001). When included in 5'-end they protect oligonucleotides from T7 Gene6 Exonuclease (Erdogan et al. 2001) and λ-exonuclease .

In a another preferred embodiment of the method of the present invention the cyclic ligation reaction of step 1(b) is effected with thermostable DNA ligase, preferably Taq DNA ligase, Tth DNA ligase, and most preferably with Pfu DNA ligase.

Comparisons with other commercially available thermostable ligases such as Taq DNA ligase, Tth DNA ligase, Ampligase and Ligase-65 showed that the Pfu DNA ligase manifests higher fidelity.

In still another preferred embodiment of the method of the present invention the concentration of DOs in each DO set is in the range of 10pM to 10nM, preferably 1nM.

In a further preferred embodiment of the method of the present invention only one cycle of ligation reaction is performed in step 1(b), preferably more than 10 cycles, and most preferably 15 to 25 cycles.

In still a further preferred embodiment of the method of the present invention the cyclic ligation of step 1(b) is effected in the presence of at least 1% polyethyleneglycol (PEG), preferably in the presence of at least 5% PEG and most preferably in the presence of about 15% PEG.

In a more preferred embodiment of the method of the present invention said polyethylene glycol has a molecular weight of more than 200, preferably more than 3000 and most preferably in the range 6000 – 8000.

Concentration of DOs is one of the main parameters in the method of the invention. Too low amount of DOs leads to the suppression of the signal, whereas too high amounts result in increase of background. Optimal intermediate concentration of DOs enough for the quantitative detection and not too high to induce background are preferred. The inventors discovered that 0.1 – 1 nM concentrations of detector oligonucleotides are enough to provide quantitative hybridization with the target in short time (20 sec during cyclic ligation) in the presence of 15% PEG 6000. About 50 times less DOs are required (2 – 20 pM) if long hybridization (for some hours) precedes the ligation. Concentration of DOs should be about ten times higher if hybridization is performed without PEG. The inventors could show that PEG with different molecular weight (from 200 to 12000) stimulates the ligation reaction (though PEG with Mw more then 3000 works better, than PEG 200 – 600).

Different DOs may have different minimal concentrations because of particular secondary structure and sequence-specific interactions with non-target genome sequences. High enough concentrations are preferred for high-throughput projects to exclude time-consuming individual optimization. Examples 1 and 4 show, that 5 fmol of detector oligonucleotides in 5µl (1nM concentration) ligation reaction give practically the same results for different loci.

In a another preferred embodiment of the method of the present invention the cyclic ligation of step 1(b) is effected with at least 2 subcycles, preferably with 3 to 6 subcycles.

Each cycle of the ligation reaction may include subcycles of denaturation of unligated DOs {65°C – 30sek, 74°C – 10sek in the Examples}. When the temperature increases to 74°C, the majority of mismatched DOs get off the template and the free templates may be occupied by matched DOs. Ligated DOs remain hybridized because they are longer and have a higher melting temperature. The subcycles (i) therefore raise the chance of matched DOs to occupy the template and be ligated,

and thus increase the yield of the reaction by about two fold; (ii) decrease the time when mismatched DOs may be ligated and as a result, misligation is decreased.

The invention also relates to a detector oligonucleotide (DO) selected from the group consisting of (a) a first DO which comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination and a Region III, for locus specific hybridization, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and (b) a second DO, which comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and (c) a third DO, which is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridization, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ teminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in

sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets;

In a preferred embodiment of the invention, said detector oligonucleotide comprises a nucleotide sequence as set forth in SEQ ID NOs 1 to 129.

Furthermore, the invention relates to the use of the detector oligonucleotide of the invention in the allele discrimination of biallelic blocks of closely located nucleotide substitutions or small insertions/deletions, wherein Region III comprises a DNA sequence which corresponds to the first allelic state of said block and Region III′ comprises a DNA sequence which corresponds to the second allelic state of said block and wherein Region III and III′ have different 3′ terminal nucleotides.

Finally, the invention relates to a kit comprising one or several detector oligonucleotide sets, wherein each set comprises a first, a second and a third detector oligonucleotide (DO), wherein said first DO comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination and a Region III, for locus specific hybridization, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and wherein said second DO comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first

allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and wherein said third DO is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridization, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ teminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets.

The figures show:

Figure 1. The maximum discrimination of ligase-detection reaction is achieved, if SNP position corresponds to the extreme 3'-end of "left" DOs (here #1 and #2 are "left" DOs, #3 is "right" DO). DOs #1 and #3 are preferentially ligated in the presence of allele 1 (T), DOs #2 and #3 – in the presence of allele 2 (G).

Figure 2. General design of detector oligonucleotides. Regions I and V (marked streaky) are used for PCR amplification. They are the same for all loci. Two common PCR primers (#PCR_L, #PCR_R) are shown in the lower part of the figure. Region II is the allele-discrimination part. Two types of sequences (marked black and white) are enough for biallelic loci. Region VI is the locus-discrimination part. n types of sequences ("type_01", "type_02", ... , "type_n") are necessary to discriminate n simultaneously ligated loci. Regions III and IV are locus specific sequences responsible for targeting DOs to the SNP positions.

Figure 3. Overview of the SNP detection protocol. Step 1: multiplex ligation. Step 2: PCR preamplification with the common primers. Step 3: locus-specific amplification with one specific PCR primer.

Figure 4. Overview of the "single locus" SNP detection protocol. Step 1: depending on the allelic state of the locus a particular "left" DO is ligated to the "right" DO. Step 2: PCR amplification.

Figure 5. Overview of microarray-based detection procedure with locus-discriminating Region VI located in "left" DOs.

Figure 6. Overview of "single locus" SNP detection protocol of Example 1. Step 1: depending on the allelic state of the locus particular allele-discrimination region is included in ligated pair of detector oligonucleotides. Step 2: PCR amplification with TaqMan detection. Sequences of TaqMan probes coincide with the sequences of allele-discrimination regions.

Figure 7 Overview of the SNP-detection protocol of Example 2. Step 1: depending on the allelic state of the locus particular allele-discrimination region is included in ligated pair of detector oligonucleotides. Step 2: PCR preamplification with common PCR primers. Step 3: TaqMan detection with universal detector oligonucleotides reveals the allelic state of each locus. Amplification is performed in *n* individual tubes. Only particular locus is amplified if one PCR primer is locus-specific (#PCR_01r, #PCR_02r, ... #PCR_nr).

Figure 8. DOs and PCR primers for locus "01" aligned relative to the genomic sequence. **A.** Genomic sequences in the vicinity of SNP position for accessions Col-0 and C24. **B.** Roman numerals indicate structure regions of DOs. **C.** Oligonucleotides used for genotyping. Locus-specific oligonucleotides used for preparation of DOs (#L1_01, #L2_01, #R_01) are marked by a gray background. #L1, #L2 and #R are common primers. #aL1, #aL2 and #aR are adaptors used for ligation-based synthesis of DOs. #fam and #tet are universal TaqMan probes. #PCR_L and #PCR_R are

common PCR primers. #PCR_01r is a locus-discrimination PCR primer used in Example 2.

Figure 9. Scatter plots of "single locus" genotyping data shown in Tables 3 and 4. Fam and Tet signals are plotted on the X and Y axes respectively. Homozygous samples are represented as circles (C24) and squares (Col-0), heterozygous (C24/Col-0) – as triangles, no DNA controls – as rhombs. **A.** Fifteen independent genotyping reactions were performed for loci "18" and "20" on 5-150ng of genomic DNA. **B.** Genotyping of 30 different loci was performed on 50ng of homozygous and heterozygous genomic DNA. Arrows show signals for loci "18" and "20".

Figure 10. Scatter plots of genotyping data shown in Tables 5 and 6. Fam and Tet signals are plotted on the X and Y axes respectively. Homozygous samples are represented as circles (C24) and squares (Col-0), heterozygous (C24/Col-0) – as triangles, no ligation controls (amplification with common PCR primers) – as rhombs. **A.** Fifteen independent genotyping reactions were performed for loci "10", "18", "20", and "29" on 5ng (filled icons), 17ng, 50ng and 150ng of genomic DNA. **B.** Genotyping of 30 different loci was performed on 50ng of homozygous and heterozygous genomic DNA. Arrows show signals for loci "10", "18", "20", and "29". Signals for locus "15" are not shown on the plot.

Figure 11. Influence of DO concentration (13pM, 40pM, 130pM, 400pM, and 1300pM) on the fluorescent signal. Genotyping was performed on 50ng of Ler DNA. Tet and Fam signals are shown as circles and squares respectively.

Figure 12. Practically all templates are used in each ligation cycle.

Figure 13. 2 hours hybridization followed by short ligation. Amplification profiles demonstrate, that ligation is quantitative for DOs concentration >20pM without PEG (A) or >2pM in presence of 15% PEG 6000 (B).

The examples illustrate the invention.

MATERIALS AND METHODS

30 Arabidopsis thaliana SNP loci were used in the work. Two loci ("25" and "26") polymorphic for accessions Columbia (Col-0) and Landsberg (Ler) are from (15). The other 28 loci (for accessions Col-0 and C24) were selected from the MASC-SNP database: http://www.mpiz-koeln.mpg.de/masc/ (Schmid et al., 2003). 24 SNPs are spread evenly along the 1st chromosome of A. thaliana and 6 are distributed randomly on the 4th chromosome. Genomic sequences adjacent to SNP positions (30nt's from both sides) are shown in Table 1.

The list of common oligonucleotides and 30 sets of locus-specific oligonucleotides is given in Table 2. The fluorescent probes (with FAM or TET on 5'-ends and TAMRA on 3'-ends) for TaqMan assay were prepared by MWG Biotech (Germany). Other oligonucleotides were synthesized by TIB Molbiol (Germany).

T4 DNA ligase, Taq DNA ligase and T4 PNK were from New England BioLabs (USA). Pfu DNA ligase was from Stratagene (USA), Tth DNA ligase – from ABgene (UK), Ampligase – from Epicentre Technologies (USA) and Ligase-65 – from MRC-Holland. Reagents for real-time PCR (TaqMan PCR Core reagents kit, #N808-0228), optical plates and covers were from Applied Biosystems (USA).

Preparation of detector oligonucleotides (DOs).

Locus-specific oligonucleotides correspondent to Regions III and IV of DOs were selected so that their melting temperatures were close to 55°C and 60°C respectively (estimated by the Vector NTI program (InforMax, USA) for 4000pM primers and 50mM salt). We tried to exclude sequences showing strong homology to other regions of the A. thaliana genome or containing tandem repeats and long single base stretches.

For ligation-based synthesis of DOs (Borodina et al. 2003) the "left" locus-specific oligonucleotides (#L1_01, ..., #L1_30, and #L2_01, ..., #L2_30) were

phosphorylated: 1 nmol of each oligonucleotide was incubated at 37°C for 1 hour in 10µl of 1x T4 PNK buffer (Tris-HCl pH 7.6, 70mM; MgCl2, 10mM; dithiothreitol, 5mM) with 1mM ATP and 2.5u of T4 PNK. The PNK was then heat inactivated at 65°C for 20 minutes. Phosphorylated primers were used in LBS without any purification. Oligonucleotide #R, which is common for all loci, was phosphorylated during the phosphoramidite synthesis.

1nmol-scale ligation reactions (involving 1 nmol of each of three oligonucleotides: common, locus-specific and adapter) were performed for 1 hour at 20°C in 30µl of 1xT4 ligase buffer (Tris-HCl pH 7.5, 50mM; MgCl2, 10mM; dithiothreitol, 10mM; ATP, 1mM; BSA, 25 µg/ml), with PEG 6000 (15%) and 250u of T4 DNA ligase. T4 DNA ligase was then heat inactivated at 65°C for 15 minutes. The "right" DOs were phosphorylated for 30 min at 37°C directly in the ligation mixture by adding 2.5u of T4 PNK.

DOs were purified through denaturing PAGE electrophoresis. Corresponding bands were visualized by UV shadowing on printer paper (or on the DC Alufolien Kieselgel 60F254 chromatographic plate (Merck, Germany) with a little bit higher sensitivity) and were cut out. DNA was eluted in 150µl of 2M LiClO4 for 1 hour at 60°C and precipitated with acetone as described in (Daniliuk et al. 1986).

Detection of a single SNP locus.

The overview of the procedure is shown in Figure 6. Ligation detection was performed on different amounts of homozygous or heterozygous Arabidopsis thaliana genomic DNA in the presence of 5fmol of each DO (one locus set per tube) in 5µl of 1x Pfu ligase buffer (Tris-HCl, pH 7.5 20mM; KCl, 20mM; MgCl2 10mM; Igepal, 0.1%; ATP, 0.01mM; DTT 1mM) with PEG 6000 (15%) and 2u of Pfu DNA ligase. The thermal profile of the reaction was: 95°C – 2min; ((65°C – 30sek, 74°C – 10sek) x6 subcycles) x20 cycles.

15µl of PCR mix (1xTaqMan Buffer, 400nM primers #PCR_L and #PCR_R, 300nM TaqMan probes #fam and #tet, and 0.5u AmpliTaq Gold polymerase) was added to 5µl of ligation reaction. The TaqMan assay with end-point fluorescent detection was

carried out on an ABI 7900HT system (Applied Biosystems, USA): 95°C – 10min, (95°C – 15sek, 60°C – 30sek) x40 cycles.

The multilocus SNP detection.

The overview of the procedure is shown in Figure 7. 30 locus-specific DO-sets were ligated simultaneously on genomic DNA (5fmol of each DO).

Preamplification of the ligated products (PCR I) was carried out with common primers #PCR_R and #PCR_L. 15µl of PCR mix (1xTaqMan Buffer, 400nM primers #PCR_R and #PCR_L, and 0.25u AmpliTaq Gold polymerase) was added to 5µl of ligation reaction and the tube was amplified in a thermocycler: 95°C – 10min, (95°C – 15sek, 60°C – 30sek) x10 cycles and then diluted with 180µl of water.

1µl of the diluted PCR I was taken as a template for the locus-specific PCR, performed in 10µl of (1xTaqMan Buffer, 400nM primers #PCR_L and one of the locus-specific primers (PCR_01r, ..., PCR_30r), 300nM TaqMan probes #fam and #tet, and 0.25u AmpliTaq Gold polymerase). The TaqMan assay with end-point fluorescent detection was carried out on an ABI 7900HT system (Applied Biosystems, USA): 95°C – 10min, (95°C – 15sek, 60°C – 30sek) x40 cycles.

Determination of minimal concentration of DOs.

Genotyping on 50ng of Ler DNA was performed with 13, 67, 130, 670 and 1300 pM locus "25" DOs. All parameters of ligation and subsequent amplification were as described in protocol of detection of a single SNP locus, except for shorter annealing time in cyclic ligation: (95°C – 2min; 55°C – 20sek) x20 cycles.

EXAMPLES

The procedures of examples 1 and 2 are outlined schematically in figures 6 and 7. In both cases the first step is a ligation detection reaction (LDR) converting the biallelic state of SNP locus into the bimarker state of ligated detector oligonucleotides (DOs). The state of the markers (Region II and II') is determined by a TaqMan assay during subsequent PCR amplification of ligated DOs.

The same DOs are used both for single and multiplex ligation (Figure 8B). Three DOs are required per each biallelic locus: a common "right" DO (DOR) and two different "left" DOs (DOL1 and DOL2).

The only locus-specific parts of DOs are Regions III and IV, which target DOs to particular SNP position. Regions III are allele-specific: they have different 3'-terminal nucleotides corresponding to the SNP position.

The other parts of the DOs (Regions I, II, II' and V) are the same for the different DO-sets. They are used for amplification combined with the TaqMan assay. Regions I and V are used in PCR amplification. The sequence of Region I is identical to the primer #PCR_L. The sequence of Region V is complementary to the primer #PCR_R. Regions II and II' (shown black and white in Figure 8B) are targets for universal TaqMan probes (#fam and #tet in Figure 8C). Region II and II' variants are arbitrary assigned to SNP alleles. In both examples the "fam"-sequence is assigned to Columbia (Col-0) alleles and the "tet"-sequence – to C24 or Landsberg (Ler) alleles.

The procedure requires rather long DOs (65-80nt for "left" DOs and 50-60nt for "right" DOs). However, the distinct block structure, i.e. combination of constant and variable parts, allows their cost-effective generation by ligation-based synthesis (LBS) (Borodina et al. 2003). The alignment of the oligonucleotides involved in LBS of DO-set for the locus "01" is shown in Figure 8C. The LBS procedure utilizes T4 DNA ligase to join the ends of the presynthesized oligonucleotides: #L1 and #L1_01; #L2 and #L2_01; #R1 and #R_01. Since T4 DNA ligase requires a double-stranded DNA substrate, these pairs are annealed to adapter oligonucleotides #aL1, #aL2 and #aR respectively. Locus-specific oligonucleotides #L1_01, #L2_01 and #R_01 have 5 nt overhangs complementary to adapters (shown bold in Table 2).

Example 1: Detection of a single SNP locus

Detection of a single SNP locus (Figure 6) is a two-step reaction with end-point fluorescent detection. The first step is the ligation detection performed on the genomic DNA. Depending on the allelic state of SNP locus one of the "left" DOs is ligated to the "right" DO forming an amplicon with universal 3'- and 5'-ends. The TaqMan assay is performed in the same tube after addition of the amplification mixture (buffer with Taq polymerase and primers). The same amplification primers and TaqMan probes are used for all loci, making the technique cost efficient and convenient. The closed-tube fluorescent detection prevents cross-contamination.

28 SNP loci polymorphic between A. thaliana accessions Col-0 and C24 and 2 loci polymorphic between accessions Col-0 and Ler were genotyped. Reproducible and reliable results were obtained for all 30 loci tested.

End-point fluorescent signals for 15 independent genotypings of loci "18" and "20" performed on different amounts of genomic DNA (5ng - 150ng) are presented in Table 3 and in Figure 9A. In this graph, the signals for the different types of DNA (homozygous Col-0, C24 and heterozygous Col-0/C24) are located in compact areas and well separated from each other (Fig. 9A).

The signals for genotyping of all loci on 50ng of DNA are plotted together in Figure 9B (Table 4). Even taken together the signals for different loci are clearly grouped in three areas. As expected, the resulting dots for heterozygous DNA are more dispersed than dots for homozygous DNAs. However, Figure 9A demonstrates, that the location of dots for each particular locus is reproducible, which is essential for automated scoring.

Example 2: Multilocus SNP detection

The overview of the multilocus SNP detection protocol is shown in Figure 7. LDR is performed in one tube simultaneously for a number of loci. The allelic state of each particular locus is determined in separate TaqMan reactions with one common and one locus-specific PCR primers (step 3 in Figure 7). Locus-specific primers

(#PCR_01r, ... , #PCR_nr) are used to distinguish single amplicon from a complex mixture. An intermediate preamplification step 2 is introduced into the protocol to prevent decrease of sensitivity due to the distribution of the ligation mixture into a number of separate tubes for the locus-specific TaqMan assay.

The procedure was tested for the parallel genotyping of the same 30 SNP loci described in Example 1. The end-point fluorescent signals for loci "10", "18", "20" and "29" obtained in fifteen independent genotyping on different amounts of genomic DNA (5ng - 150ng) are shown in Table 5 and Figure 10A. For all loci signals for different types of DNA (homozygous Col-0, C24 and heterozygous Col-0/C24) are well separated and are tightly grouped for ≥17ng of genomic DNA.

The fluorescent signals for genotyping of all loci together on 50ng of genomic DNA are shown in Table 6 and are plotted in Figure 10B. Again, areas of different genotypes are reliably separated from each other.

Some DO-sets may be incompatible with others in a parallel analysis, since some particular DO may serve as a template for misligation of other DOs. Among 30 loci tested only one locus (number "15") showed such a problem. Though the DO-set for locus "15" works excellently in the "single locus genotyping" (Table 4) it gives a "Fam/Tet" ratio about 4 in multilocus reaction on heterozygous DNA (Table 6).

The main point of the parallel analysis is that in this format considerably less genomic DNA is required for genotyping of each particular locus: 17ng DNA for genotyping of 30 loci means 0.6ng per locus, and we have clearly not reached the limit of multiplexing in these tests.

Example 3. Minimal Concentration of DOs

To estimate the minimal possible concentration of DOs in cyclic hybridization/ligation reaction, genotyping on 50ng of Landsberg Arabidopsis DNA was performed with 13 - 1300 pM DOs (set "25"). In these experiments the time of hybridization/ligation was 20 sec. All other parameters of ligation and subsequent amplification were as described in Example 1. Ligation detection was performed in the presence of various

amounts of linear DOs (13 –1300pM) in 5µl of 1x Pfu ligase buffer (Tris-HCl, pH 7.5 20mM; KCl, 20mM; MgCl2 10mM; Igepal, 0.1%; ATP, 0.01mM; DTT 1mM) with 15% PEG 6000 and 2u of Pfu DNA ligase. Thermal profile of the reaction was the following: (95°C – 2min; 55°C – 20sek)x20 cycles. The results are shown in Figure 11. Concentrations of DOs > 130 pM s are enough for quantitative hybridisation in 20 sec. When 13 – 130pM concentrations of DOs are used, the signal decreases, but is still sufficient for the reliable detection.

Comparison of real-time amplification profiles of (i) material obtained in 20 cycles of ligation on 100ng of Arabidopsis genomic DNA with DO-set "25" and (ii) known amount of ligated DOs (40 amol) showed that practically all templates are used in each cycle (Figure 12). The theoretically possible amount after ligation is 88amol (22[amol]/1[µg] x 100[ng] x 20[cycles] x 2[alleles]). The known amount of the ligated product was prepared by ligation of "left" DO2_25 and "right" DO_25 (2 fmol each) on 1.3 fmol of template #tmpl_25 for 20 cycles of (94°C-10sec; 55°C-3min). The reaction was performed in 5µl of 1xTth ligase buffer (TrisHCl 20mM, pH8.3; MgCl2 10mM; KCl 50mM; EDTA 1mM; NAD+ 1mM; DTT 10mM; Triton X-100 0.1%) in the presence of 50ng D.melanogaster genomic DNA.

Longer hybridisation time results in decrease of the minimal concentration of DOs. Two hours hybridisation followed by 5min ligation was performed on the constant temperature (without subcycles). About the same signals were obtained for concentrations of DOs >20pM (without PEG) and >2pM (with 15% PEG 6000) (Figure 13). When 2 – 20pM concentration of DOs is used (without PEG) the signal decreases, but is still sufficient for the reliable detection (Table 7). Hybridisation was performed in the presence of 2-200pM of linear DOs in 5µl of 1x Pfu ligase buffer (Tris-HCl, pH 7.5 20mM; KCl, 20mM; MgCl2 10mM; Igepal, 0.1%; ATP, 0.01mM; DTT 1mM) with (or without) 15% PEG 6000. Thermal profile of the reaction was the following: (95°C – 2min; 55°C – 2hours). Then 2u of Pfu DNA ligase were added and the mixture was incubated 5 min at 55°C. Real time detection with SybrGreen II was performed on ABI 9700HT system.

Another conclusion from Figure 13 is that 15% PEG 6000 decreases the minimal concentration of detector oligonucleotides in about ten times. The same effect was observed for shorter hybridization times.

REFERENCES

Barany, F., Lubin, and M., Belgrader, P. 2001. Detection of nucleic sequence differences using coupled ligase detection and polymerase chain reaction. Unated States Patent 6,268,148.

Borodina, T.A., Lehrach, H., and Soldatov, A.V. 2003. Ligation-based synthesis of oligonucleotides with block structure. Anal Biochem. 318: 309-313.

Brookes, A.J. 1999. The essence of SNPs. Gene 234:177-86.

Chen, X., Levine, L., and Kwok, P.Y. 1999. Fluorescence polarization in homogeneous nucleic acid analysis. Genome Res. 9:492-8.

Cho, R.J., Mindrinos, M., Richards, D.R., Sapolsky, R.J., Anderson, M., Drenkard, E., Dewdney, J., Reuber, T.L., Stammers, M., Federspiel, N., et al. 1999. Genome-wide mapping with biallelic markers in Arabidopsis thaliana. Nat Genet. 23:203-7.

Crow, J.F. 1995. Spontaneous mutation as a risk factor. Exp. Clin. Immunogenet. 12:121-128.

Dean, F.B., Nelson, J.R., Giesler, T.L., and Lasken, R.S. 2001. Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. Genome Res. 11:1095-9.

Daniliuk, N.K., Iastrebov, S.I., Artamonova, T.P., and Popov, S.G. 1986. A simplified variant of the Maxam-Gilbert method for determining the primary structure of oligonucleotides and DNA fragments. Bioorg Khim. .12:1185-8.

Erdogan, F., Kirchner, R., Mann, W., Ropers, H.H., and Nuber, U.A. 2001. Detection of mitochondrial single nucleotide polymorphisms using a primer elongation reaction on oligonucleotide microarrays. Nucleic Acids Res. 29:e36.

Faruqi, A.F., Hosono, S., Driscoll, M.D., Dean, F.B., Alsmadi, O., Bandaru, R., Kumar, G., Grimwade, B., Zong, Q., Sun, Z., et al. 2001. High-throughput

genotyping of single nucleotide polymorphisms with rolling circle amplification. BMC Genomics 2:4.

Hardenbol, P., Baner, J., Jain, M., Nilsson, M., Namsaraev, E.A., Karlin-Neumann, G.A., Fakhrai-Rad, H., Ronaghi, M., Willis, T.D., Landegren, U., Davis, R.W. (2003) Multiplexed genotyping with sequence-tagged molecular inversion probes. Nat Biotechnol., 21, 673-678.

Hawkins, J.R., Khripin, Y., Valdes, A.M., and Weaver, T.A. 2002. Miniaturized sealed-tube allele-specific PCR. Hum Mutat. 19:543-53.

Holland, P.M., Abramson, R.D., Watson, R., and Gelfand, D.H. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 88:7276-80.

Howell, W.M., Jobs, M., Gyllensten, U., and Brookes, A.J. (1999) Dynamic allele-specific hybridization. A new method for scoring single nucleotide polymorphisms. Nat Biotechnol., 17, 87-88.

Li, Q., Luan, G., Guo, Q., and Liang, J. 2002. A new class of homogeneous nucleic acid probes based on specific displacement hybridization. Nucleic Acids Res. 30:e5.

Li, W.H., Ellsworth, D.L., Krushkal, J., Chang, B.H., and Hewett-Emmet, D. 1996. Rates of nucleotide substitution in primates and rodents and the generation-time effect hypothesis. Mol. Phylogenet. Evol. 5:182-187.

Li, W.H., and Sadler, L.A. (1991) Low nucleotide diversity in man. *Genetics,* 129, 513-523.

Livak, K.J., Marmaro, J., and Todd, J.A. 1995. Towards fully automated genome-wide polymorphism screening. Nat Genet. 9:341-2.

Lizardi, P.M., Huang, X., Zhu, Z., Bray-Ward, P., Thomas, D.C., and Ward, D.C. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. Nat Genet. 19:225-32.

Lutz, S., Ostermeier, M., and Benkovic, S.J. 2001. Rapid generation of incremental truncation libraries for protein engineering using alpha-phosphothioate nucleotides. Nucleic Acids Res. 29:e16.

Lyamichev, V., and Neri, B. (2003) Invader assay for SNP genotyping. Methods Mol Biol., 212, 229-240.

Myakishev, M.V., Khripin, Y., Hu, S., and Hamer, D.H. (2001) High-throughput SNP genotyping by allele-specific PCR with universal energy-transfer-labeled primers. Genome Res., 11, 163-169.

Prince, J.A., Feuk, L., Howell, W.M., Jobs, M., Emahazion, T., Blennow, K., and Brookes, A.J. (2001) Robust and accurate single nucleotide polymorphism genotyping by dynamic allele-specific hybridization (DASH): design criteria and assay validation. Genome Res., 11, 152-162.

Schmid KJ, Sorensen TR, Stracke R, Torjek O, Altmann T, Mitchell-Olds T, Weisshaar B. (2003) Large-scale identification and analysis of genome-wide single-nucleotide polymorphisms for mapping in Arabidopsis thaliana. Genome Res. 13:1250-7.

Tost, J., and Gut, I.G. (2002) Genotyping single nucleotide polymorphisms by mass spectrometry. Mass Spectrom Rev., 21, 388-418.

Tyagi, S., Bratu, D.P., and Kramer, F.R. 1998. Multicolor molecular beacons for allele discrimination. Nat Biotechnol. 16:49-53.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., et al. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23:4407-14.

Wang, D.G., Fan, J-B., Siao, C-J., Berno, A., Young, P., Sapolsky, R., Ghandour, G., Perkins, N., Winchester, E., Spencer, J., et al. (1998) Large scale identification, mapping, and genotyping of single nucleotide polymorphisms in the human genome. Science, 280, 1077-1082.

Tables.

Table 1

Genomic sequences of SNP loci analysed in Examples 1-3 (30nt's from both sides of SNP position). Sequences of loci "25" and "26" are from (Cho et al. 1999).

Locus	Chr. Position	MASC Number	Genomic sequences	
			Chromosome 1	
1	0.85_M	MASC03692	CCCCAAAGTTCGTAGATGAAAATGGTAACA C GAGGACTTAAAACAAGAAAGTTCCTCGCAA	(Col-0)
			CCCCAAAGTTCGTAGATGAAAATGGTAACA A GAGGACTTAAAACAAGAAAGTTCCTCGCAA	(C24)
2	2.04_M	MASC06796	ATAGTTTGGTCTTTTATATATCACAGATTG T AGACAGGCATTACTCAAAGTGAAGCCATGT	(Col-0)
			ATAGTTTGGTCTTTTATATATCACAGATTG C AGACAGGCATTACTCAAAGTGAAGCCATGT	(C24)
3	2.69_M	MASC02113	TTTTCGAAAATTACACCAGATTCACCAAAC G CGACGCGCCTCTTCCTTCTTGGTCTTCCTC	(Col-0)
			TTTTCGAAAATTACACCAGATTCACCAAAC C CGACGCGCCTCTTCCTTCTTGGTCTTCCTC	(C24)
4	4.10_M	MASC07144	CAGAAGCAAGAGACAAAGTCATGGACGAAG G AGACACACATTTCATGTTGGTTTGTAGTAT	(Col-0)
			NNNNNGCAAGAGACAAAGTCATNGACGAAG A AGACACACATTTCATGTTGGTTTGTAGTAT	(C24)
5	5.40_M	MASC00817	AGTTGAGACCCTCTCTGGTGTCCTCGCCGG A TCTCTTGTATCCGGTGTTCAGGTAAAGTAT	(Col-0)
			AGTTGAGACCCTCTCTGGTGTCCTCGCCGG C TCTCTTGTATCCGGTGTTCAGATCGCCATA	(C24)
6	6.82_M	MASC06752	ACCTGGTTCCACCGACGCTTACAGTTATCC T CTAAAGAGTTGCAAATACATATTCATGCTC	(Col-0)
			ACCTGGTTCCACCGACGCTTACAGTTATCC A CTAAAGAGTTGCAAATACATATTCATGCTC	(C24)
7	7.83_M	MASC02593	TTTAGAAGGTAAAGACAAATGTGCTCTTGA T GGGAAACAAACCATCAAATCTGCTGAGTAA	(Col-0)
			TTTAGAAGGTAAAGACAAATGTGCTCTTGA A GGGAAACAAACCATCAAATCTGCTGAGTAA	(C24)
8	8.69_M	MASC07163	AAGCCAAAGGTTAGGACAAAGGTAGCAACA A ATCAGTATTCAGAATTGGAGATGACATCGT	(Col-0)
			AAGCCAAAGGGTAGNANAATGGTAGCAACA C ATCAGTATTCAGAANNNNAGATGACATCGT	(C24)
9	9.84_M	MASC06722	TATTTCAGAGTTGTGTAGTGTAGTTGCAAG T GGCGGTTGGATCTTTCACTATCTTGTTGGA	(Col-0)
			TATTTCAGAGTTGTGTAGTGTAGTTGCAAG C GGCGGTTGGATCTTTCACTATCTTGTTGGA	(C24)
10	10.70_M	MASC02175	TCGAAGACCATTGAACTGAGGCAATGTAGT C ATCATAGTGCTAGCCATTTTCTTAGTTGAT	(Col-0)
			TCGAAGACCATTGAACTGAGGCAATGTAGT T ATCATAGTGCTAGCCATTTTCTTAGTTGAT	(C24)
11	11.91_M	MASC04228	TTAAAAATGGGATAACATTTTAGTGCATAA C AAAGTTTTAGACTTAAGGTTGAAAAAACGA	(Col-0)
			TTAAAAATGGGATAACATTTTAGTGCATAA G AAAGTTTTAGACTTAAGGTTGAAAAAACGA	(C24)
12	13.07_M	MASC00364	GACGACTGATTCCGCGAATTTCGGAGCGAC G GTGAATGCGGTTCGAGACGTCGACGCAGTG	(Col-0)
			GACGACTGATTCAGCGAATTTCGGAGCGAC A GTGAATGCGGTTCGAGACGTCGACGCAGTG	(C24)
13	15.37_M	MASC04127	GAGCATCTTACAGCAGCTCAAACTTCAAAT A TCGTAGTGCTGAGCATTAAGTACCTGCGAT	(Col-0)
			GAGCATCTTACAGCAGCTCAAACTTCAAAT G TCGTAGTGCTGAGCATTAAGTACCTGCGAT	(C24)
14	16.35_M	MASC06782	TTGAGAGATACCTTGTTCTTTGAGACCGAG G GCAGATCGGACGTTTTTGCCGGGAGGAATG	(Col-0)
			AAACAATGGACCTTGTTCTTTGAGACCGAG A GCAGATCGGACGTTTTTGCCGGGAGGAATG	(C24)
15	17.66_M	MASC05011	GACAAACAAGAGAAGCGGAGAAAGTGAGAC G GTAAGGATCTCTCCGGTGTCGGCTTTTGTT	(Col-0)
			GACAAACAAGAGAAGCGGAGAAAGTGANAC A GTAAGGATCTCTCCGGTGTCGGCTNNNNNN	(C24)
16	18.72_M	MASC06707	CCTACCGCCAAGATATGCCGTAACTGAGTT T GCTATTTAACCAGCAACTGTATCTATGTCG	(Col-0)
			CCTACCGCCAGGATATGCCGTAACTGAGTT C GCTATTTAACCAGCAACTGTATCTATGTCG	(C24)
17	19.76_M	MASC00330	TCCTCCCACCTCATCAAGCTTCGTCAGGAA T GTCCACTCGGATGTCACCGTATCGTAGGAG	(Col-0)
			TCCTCCCACCTCATCAAGCTTCGTCAGGAA C GTCCACTCGGATGTCACCGTATCGTAGGAG	(C24)
18	20.75_M	MASC07052	GTCGTGATGTCACTTAACCATCATCAACCC T TCGAATTAGTTCCTAGAAACCTATTATATC	(Col-0)
			GNCGTNGTGTCACTNNNNNNTCATCAACCC A TCGAATTAGTNNNNNNNNNNNTNNNNNNNN	(C24)

19	21.93_M	MASC04194	TGATTTCTAAGCCACTGTGAGTGCCCTTGA C TTGATTATATATTCGGATTGTGTTGTTTTC (Col-0) TGATTTCTAAGCCACTGTGAGTGCCCTTGA T TTGATTATATATTCGGATTGTGTTGTTTTC (C24)
20	23.17_M	MASC02688	AGCAGAACGAAGAGAGTAACGAACGATGAC T GGTTTACTTTCAAATTCAGAGAAGAAGGAA (Col-0) AGCAGAACGAAGAGAGTAACGAACGATGAC C GGTTTACTTTCAAATTCAGAGAAGAAGGAA (C24)
21	25.41_M	MASC06750	CGAAAGATGGTGAGTTTTTGAGACACAACT A GGATATTTCTTAATGGTTTGTGCCACTAAT (Col-0) CGAAAGATGGTGAGTTTTTGAGACACAACT G GGATATTTCTTAATGGTTTGTTGCCACTAA (C24)
22	26.79_M	MASC00629	GTACCAAGCTCTCAAGATGTCTCTCGGTGT A TTCACCGCTTTCAGCATCGGTGTTGGGGTT (Col-0) GTACCAAGCTCTCAAGATGTCTCTCGGTGT C TTCACCGCTTTCAGCATCGGTGTTGGGGTT (C24)
23	28.02_M	MASC07087	AGAGTCAATCCATGTTATTGTTCTTGGTTT C TCTGCTGCTGTCATTACAGACGTTGATATC (Col-0) NNANNCAATCCATGTTATTGTTCTTGGTTT T TCTGCTNNNGTCATTACAGACGTTGATATC (C24)
24	29.09_M	MASC07132	ATCGGCTTGGGCTTACACGGTGGCGCAATT T AGCTGGCCGTTCCAGTGGAGTCAGCGGGTC (Col-0) ATCGGCTTGGGCTTACACGGTGGCGCAATT G AGCTGGCCGTTCCAGTGGAGTCAGCGGGTC (C24)
		Chromosome 4	
25*	12.0_M	SNP149	AAGCAAGCGGCGAACATTTAAGACCAAACT G TTGTAAAACGTCGGGAGAAACAGAGAGCGA (Col-0) AAGCAAGCGGCGAACATTTAAGACCAAACT A TTGTAAAACGTCGGGAGAAACAGAGAGCGA (Ler)
26*	16.2_M	SNP200	ATACGACGGAGCTAAATCGAGATCGTTAAA C CCTCCGAAGACTTCACGGTAGTCAAAACCT (Col-0) ATACGACGGAGCTAAATCGAGATCGTTAAA A CCTCCGAAGACTTCACGGTAGTCAAAACCT (Ler)
27	0.03_M	MASC02820	AGTTGTGGTAATAACAAAGGTAAGAGGGGT T AGCCATAGGAGCTCCAATCTGAGAGTGAGG (Col-0) AGTTGTGGTAATAACAAAGGTAAGAGGGGT C AGCCATAGGAGCTCCAATCTGAGAGTGAGG (C24)
28	0.5_M	MASC02948	TATCTTCAACATCACATCTGTTATTTCCTC G ATAGTCGCCTTTCGGTTCTCCTCATAGTTC (Col-0) TATCTTCAACATCACATCTGTTATTTCCTC C ATAGTCGCCTTTCGGTTCTNNNNATAGTTC (C24)
29	4.15_M	MASC04672	TATGCACCATCTATCTCTGTTTTCAGAATA T ATCTCCAGCTATCAATTAAAACCCCAAAAA (Col-0) TATGCACCATCTATCTCTGTTTTCAGAATA C ATCTCCAGCTATCAATTAAAACCCCAAAAA (C24)
30	5.1_M	MASC02658	TTGCGGTGGAACAGCAAAATGTTTCTAGTC G ACAACCTTGTTCAGATGTTTCGCTTCTTCT (Col-0) TTGCGGTGGAACAGCAAAATGTNTCTAGTC C ACAACCTTGTTCAGATGTTTCGCTTCTTCT (C24)

Table 2

Sequences of oligonucleotides used in Examples 1-3 (5' → 3' oriented). 5'- and 3'-end pentanucleotides for preparation of DOs by ligation-based method are marked bold. Alignment of locus "01" DO-set is shown in Figure 8. Genomic sequences in the vicinity of SNPs are shown in Table 1. DOR_25 and DOR_26 were prepared by conventional synthesis, so they do not contain overhangs for ligation.

Locus	Name	Oligo Sequence	Length
Locus-specific primers			
1	#L1_01	**ACGGG**AACTTTCTTGTTTTAAGTCCTCG	28
	#L2_01	**ACGGG**AACTTTCTTGTTTTAAGTCCTCT	28
	#R_01	TGTTACCATTTTCATCTACGAACTT**CGGTC**	30
	#PCR_01r	CGAAGTTCGTAGATGAAAATGGTAACA	27
2	#L1_02	**ACGGG**TTCACTTTGAGTAATGCCTGTCTA	29
	#L2_02	**ACGGG**TTCACTTTGAGTAATGCCTGTCTG	29
	#R_02	CAATCTGTGATATATAAAAGACCAAACTAT**CGGTC**	35
	#PCR_02r	GACCGATAGTTTGGTCTTTTATATATCACA	30
3	#L1_03	**ACGGG**AATTACACCAGATTCACCAAACG	28
	#L2_03	**ACGGG**AATTACACCAGATTCACCAAACC	28
	#R_03	CGACGCGCCTCTTCCTT**CGGTC**	22
	#PCR_03r	TATCCCGTTAGACCGAAGGAAGA	23
4	#L1_04	**ACGGG**AGACAAAGTCATGGACGAAGG	26
	#L2_04	**ACGGG**AGACAAAGTCATGGACGAAGA	26
	#R_04	AGACACACATTTCATGTTGGTTTGT**CGGTC**	30
	#PCR_04r	ACAAACCAACATGAAATGTGTGTCT	25
5	#L1_05	**ACGGG**CCTGAACACCGGATACAAGAGAT	28
	#L2_05	**ACGGG**TCTGAACACCGGATACAAGAGAG	28
	#R_05	CCGGCGAGGACACCAGAG**CGGTC**	23
	#PCR_05r	GTTAGACCGCTCTGGTGTCCT	21
6	#L1_06	**ACGGG**CACCGACGCTTACAGTTATCCT	27
	#L2_06	**ACGGG**CACCGACGCTTACAGTTATCCA	27
	#R_06	CTAAAGAGTTGCAAATACATATTCATGCT**CGGTC**	34
	#PCR_06r	GAGCATGAATATGTATTTGCAACTCTTTAG	30
7	#L1_07	**ACGGG**TAAAGACAAATGTGCTCTTGAT	27
	#L2_07	**ACGGG**TAAAGACAAATGTGCTCTTGAA	27
	#R_07	GGGAAACAAACCATCAAATCTGCT**CGGTC**	29
	#PCR_07r	GCAGATTTGATGGTTTGTTTCCC	23
8	#L1_08	**ACGGG**TTAGGACAAAGGTAGCAACAA	26
	#L2_08	**ACGGG**GTAGGACAATGGTAGCAACAC	26
	#R_08	ATCAGTATTCAGAATTGGAGATGACAT**CGGTC**	32
	#PCR_08r	GATGTCATCTCCAATTCTGAATACTGAT	28
9	#L1_09	**ACGGG**TGAAAGATCCAACCGCCA	23
	#L2_09	**ACGGG**TGAAAGATCCAACCGCCG	23
	#R_09	CTTGCAACTACACTACACAACTCTGAAATA**CGGTC**	35
	#PCR_09r	TTAGACCGTATTTCAGAGTTGTGTAGTGTA	30
10	#L1_10	**ACGGG**CATTGAACTGAGGCAATGTAGTC	28
	#L2_10	**ACGGG**CATTGAACTGAGGCAATGTAGTT	28
	#R_10	ATCATAGTGCTAGCCATTTTCTTAGTTGAT**CGGTC**	35
	#PCR_10r	TCAACTAAGAAAATGGCTAGCACTATGAT	29

11	#L1_11	**ACGGG**ATGGGATAACATTTTAGTGCATAAC	30
	#L2_11	**ACGGG**ATGGGATAACATTTTAGTGCATAAG	30
	#R_11	AAAGTTTTAGACTTAAGGTTGAAAAAACGA**CGGTC**	35
	#PCR_11r	CCGTCGTTTTTTCAACCTTAAGTCTA	26
12	#L1_12	**ACGGG**CGTCTCGAACCGCATTCACC	25
	#L2_12	**ACGGG**CGTCTCGAACCGCATTCACT	25
	#R_12	GTCGCTCCGAAATTCG**CGGTC**	21
	#PCR_12r	CGCGAATTTCGGAGCGAC	18
13	#L1_13	**ACGGG**CTTACAGCAGCTCAAACTTCAAATA	30
	#L2_13	**ACGGG**CTTACAGCAGCTCAAACTTCAAATG	30
	#R_13	TCGTAGTGCTGAGCATTAAGTACCT**CGGTC**	30
	#PCR_13r	CGTTAGACCGAGGTACTTAATGCTCA	26
14	#L1_14	**ACGGG**CCTTGTTCTTTGAGACCGAGG	26
	#L2_14	**ACGGG**CCTTGTTCTTTGAGACCGAGA	26
	#R_14	GCAGATCGGACGTTTTTGC**CGGTC**	24
	#PCR_14r	GGCAAAAACGTCCGATCTG	19
15	#L1_15	**ACGGG**AAGCGGAGAAAGTGAGACG	24
	#L2_15	**ACGGG**AAGCGGAGAAAGTGAGACA	24
	#R_15	GTAAGGATCTCTCCGGTGT**CGGTC**	24
	#PCR_15r	CGACACCGGAGAGATCCTTAC	21
16	#L1_16	**ACGGG**CAAGATATGCCGTAACTGAGTTT	28
	#L2_16	**ACGGG**CAGGATATGCCGTAACTGAGTTC	28
	#R_16	GCTATTTAACCAGCAACTGTATCTATG**CGGTC**	32
	#PCR_16r	CGCATAGATACAGTTGCTGGTTAAATA	27
17	#L1_17	**ACGGG**TCATCAAGCTTCGTCAGGAAT	26
	#L2_17	**ACGGG**TCATCAAGCTTCGTCAGGAAC	26
	#R_17	GTCCACTCGGATGTCAC**CGGTC**	22
	#PCR_17r	TAGACCGGTGACATCCGAGT	20
18	#L1_18	**ACGGG**TCACTTAACCATCATCAACCCT	27
	#L2_18	**ACGGG**TCACTTAACCATCATCAACCCA	27
	#R_18	TCGAATTAGTTCCTAGAAACCTATTATAT**CGGTC**	34
	#PCR_18r	CTATCCCGTTAGACCGATATAATAGGTTTC	30
19	#L1_19	**ACGGG**CCACTGTGAGTGCCCTTGAC	25
	#L2_19	**ACGGG**CCACTGTGAGTGCCCTTGAT	25
	#R_19	TTGATTATATATTCGGATTGTGTTGTTTT**CGGTC**	34
	#PCR_19r	GAAAACAACACAATCCGAATATATAATCAA	30
20	#L1_20	**ACGGG**CGAAGAGAGTAACGAACGATGACT	29
	#L2_20	**ACGGG**CGAAGAGAGTAACGAACGATGACC	29
	#R_20	GGTTTACTTTCAAATTCAGAGAAGAAGGA**CGGTC**	34
	#PCR_20r	CGTCCTTCTTCTCTGAATTTGAAAGTA	27
21	#L1_21	**ACGGG**CACAAACCATTAAGAAATATCCT	28
	#L2_21	**ACGGG**CAACAAACCATTAAGAAATATCCC	29
	#R_21	AGTTGTGTCTCAAAAACTCACCATCTTT**CGGTC**	33
	#PCR_21r	CGAAAGATGGTGAGTTTTTGAGACA	25
22	#L1_22	**ACGGG**CTCTCAAGATGTCTCTCGGTGTA	28
	#L2_22	**ACGGG**CTCTCAAGATGTCTCTCGGTGTC	28
	#R_22	TTCACCGCTTTCAGCAT**CGGTC**	22
	#PCR_22r	CCGTTAGACCGATGCTGAAAG	21
23	#L1_23	**ACGGG**AATCCATGTTATTGTTCTTGGTTTC	30
	#L2_23	**ACGGG**AATCCATGTTATTGTTCTTGGTTTT	30

	#R_23	TCTGCTGCTGTCATTACAGACGTTCGGTC	29
	#PCR_23r	CCGAACGTCTGTAATGACAGCA	22
24	#L1_24	ACGGGACACGGTGGCGCAATTT	22
	#L2_24	ACGGGACACGGTGGCGCAATTG	22
	#R_24	AGCTGGCCGTTCCAGTGGCGGTC	23
	#PCR_24r	CCACTGGAACGGCCAGCT	18
25	#L1_25	ACGGGCGGCGAACATTTAAGACCAAACTG	29
	#L2_25	ACGGGCGGCGAACATTTAAGACCAAACTA	29
	DOR_25	TTGTAAAACGTCGGGAGAAACAGAGAGGTCTAACGGGATAGCGTG	45
	#PCR_25r	TCTCTGTTTCTCCCGACGTTTTACAA	26
	#tmpl_25	CTCTCTGTTTCTCCCGACGTTTTACAACAGTTTGGTCTTAAATGTT CGCCGC	52
26	#L1_26	ACGGGAGCTAAATCGAGATCGTTAAAC	27
	#L2_26	ACGGGAGCTAAATCGAGATCGTTAAAA	27
	DOR_26	CCTCCGAAGACTTCACGGTAGTCAGTCTAACGGGATAGCGTG	42
	#PCR_26r	CGGTTTTGACTACCGTGAAGTCT	23
27	#L1_27	ACGGGAGATTGGAGCTCCTATGGCTA	26
	#L2_27	ACGGGAGATTGGAGCTCCTATGGCTG	26
	#R_27	ACCCCTCTTACCTTTGTTATTACCACACGGTC	30
	#PCR_27r	TGTGGTAATAACAAAGGTAAGAGGGGT	27
28	#L1_28	ACGGGAACCGAAAGGCGACTATC	23
	#L2_28	ACGGGAACCGAAAGGCGACTATG	23
	#R_28	GAGGAAATAACAGATGTGATGTTGAAGATCGGTC	34
	#PCR_28r	TCTTCAACATCACATCTGTTATTTCCTC	28
29	#L1_29	ACGGGTTTTAATTGATAGCTGGAGATA	27
	#L2_29	ACGGGTTTTAATTGATAGCTGGAGATG	27
	#R_29	TATTCTGAAAACAGAGATAGATGGTGCACGGTC	33
	#PCR_29r	TGCACCATCTATCTCTGTTTTCAGAATA	28
30	#L1_30	ACGGGAACAGCAAAATGTTTCTAGTCG	27
	#L2_30	ACGGGAACAGCAAAATGTTTCTAGTCC	27
	#R_30	ACAACCTTGTTCAGATGTTTCGCTTCGGTC	27
	#PCR_30r	CGAAGCGAAACATCTGAACAAG	22
Common oligonucleotides			
#L1		TGCACAATTCACGACTCACGATCCACACGGTCTCGCACTGGC	42
#aL1		CCCGTGCCAGTGCGA	15
#L2		TGCACAATTCACGACTCACGATCATCCGCTCCGACGACACGA	42
#aL2		CCCGTTCGTGTCGTCG	16
#R		TAACGGGATAGCGTGGTGGTA	21
#aR		GCTATCCCGTTAGACCG	17
#PCR_L		GCACAATTCACGACTCACGA	20
#PCR_R		CCACCACGCTATCCCGTTAGAC	22
#fam		FAM-CCACACGGTCTCGCACTGGC-TAMRA	20
#tet		TET-CATCCGCTCCGACGACACGA-TAMRA	20

Table 3

"Single locus" SNP-genotyping on different amounts of Arabidopsis genomic DNA (Example 1). Genotyping was performed either on homozygous (Col-0, C24) or heterozygous (Col-0/C24) DNA. Results from Table 4 are also included (marked by asterisk).

Locus	DNA (ng)	Col-0		C24		Col-0l/C24	
		Fam	Tet	Fam	Tet	Fam	Tet
18	150	2,65	0,14	0,43	2,57	2,20	1,12
	50	3,24	0,12	0,43	2,69	2,53	1,42
	50*	2,54	0,13	0,33	1,88	2,50	1,13
	17	3,24	0,12	0,48	2,80	2,46	1,15
	5	3,20	0,13	0,46	2,84	2,53	1,16
20	150	3,14	0,16	0,26	2,53	1,85	1,74
	50	3,20	0,23	0,47	3,06	2,22	2,01
	50*	2,68	0,17	0,34	2,32	2,34	2,12
	17	3,04	0,26	0,46	2,90	2,10	1,84
	5	3,02	0,35	0,44	2,90	2,13	2,80

Table 4

"Single locus" SNP-genotyping on 50ng of Arabidopsis genomic DNA (Example 1). Genotyping was performed either on homozygous (Col-0, C24) or heterozygous (Col-0/C24) DNA. "k" is control without DNA.

Locus	Col-0		C24		Col-0/C24	
	Fam	Tet	Fam	Tet	Fam	Tet
1	2,47	0,16	0,34	2,10	2,37	2,14
2	2,40	0,19	0,35	2,15	1,90	2,42
3	2,60	0,12	0,35	2,14	2,44	1,95
4	2,89	0,12	0,39	2,46	2,83	2,10
5	2,69	0,14	0,50	2,34	2,58	2,43
6	2,39	0,19	0,37	2,27	2,28	2,32
7	2,56	0,21	0,31	2,34	2,48	2,55
8	2,62	0,33	0,36	2,51	2,86	2,51
9	2,48	0,18	0,34	2,13	2,29	1,97
10	2,68	0,21	0,47	2,24	2,31	2,09
11	2,57	0,12	0,32	2,13	2,19	2,11
12	2,81	0,23	0,37	2,31	2,55	2,17
13	2,55	0,13	0,30	2,45	2,18	2,38
14	2,43	0,26	0,34	2,27	2,19	2,36
15	2,64	0,22	0,51	2,27	2,77	1,61
16	2,46	0,36	0,27	2,55	2,23	2,54
17	2,69	0,30	0,35	2,38	2,37	2,03
18	2,54	0,13	0,33	1,88	2,50	1,13
19	2,73	0,17	0,47	2,19	2,52	1,58
20	2,68	0,17	0,34	2,32	2,34	2,12
21	2,59	0,19	0,28	2,23	2,35	2,03
22	2,61	0,32	0,29	2,55	2,30	2,35
23	2,37	0,51	0,25	2,53	2,03	2,06
24	2,45	0,17	0,34	2,44	2,29	2,38
25	n/a	n/a	n/a	n/a	2,50	0,19
26	2,39	0,17	n/a	n/a	2,87	0,18
27	2,23	0,22	n/a	n/a	2,04	1,95
28	2,53	0,19	0,36	2,22	2,07	2,30
29	2,38	0,46	n/a	n/a	2,04	2,35
30	2,65	0,25	n/a	n/a	1,98	2,34
k	0,23	0,16	0,21	0,18	0,23	0,21

Table 5

SNP- genotyping on different amount of Arabidopsis genomic DNA (Example 2). Results from Table 6 are also included (marked by asterisk).

Locus	DNA (ng)	Col-0		C24		Col-0/C24	
		Fam	Tet	Fam	Tet	Fam	Tet
10	150	3,64	0,25	0,94	2,93	2,92	1,70
	50	3,90	0,14	1,02	3,13	3,27	1,99
	50*	3,40	-0,01	0,83	3,10	3,20	2,12
	17	3,85	0,11	1,04	2,98	3,15	1,81
	5	3,75	0,06	1,07	2,96	3,19	1,82
18	150	3,75	0,01	0,76	3,07	3,05	1,70
	50	3,61	0,09	0,77	3,01	2,90	1,80
	50*	3,26	-0,05	0,64	2,97	3,47	1,47
	17	3,46	0,16	0,73	3,06	2,97	1,43
	5	2,68	0,20	0,48	2,12	1,92	1,79
20	150	3,99	0,10	0,54	2,94	1,82	1,98
	50	3,76	0,18	0,67	3,07	2,38	2,20
	50*	3,36	-0,05	0,56	3,32	3,08	2,72
	17	3,75	0,24	0,70	2,95	2,38	2,02
	5	3,63	0,26	0,94	2,92	2,58	2,07
29	150	3,91	0,48	0,76	3,34	2,10	2,96
	50	3,76	0,57	0,70	3,48	2,12	3,21
	50*	3,65	0,01	0,65	3,49	2,29	3,27
	17	3,57	0,61	0,70	3,59	2,07	3,22
	5	2,11	0,80	0,56	3,24	2,09	2,95

Table 6

SNP-genotyping on 50ng of Arabidopsis genomic DNA (Example 2). Genotyping was performed either on homozygous (Col-0, C24) or heterozygous (Col-0/C24) DNA. "k" is control without ligation (amplification with common PCR primers).

Locus	Col-0		C24		Col-0/C24	
	Fam	Tet	Fam	Tet	Fam	Tet
1	2,99	0,06	0,65	3,18	2,96	2,36
2	3,68	0,61	0,82	3,38	2,82	3,06
3	2,07	0,19	0,52	2,15	1,94	1,52
4	4,04	-0,08	0,69	3,73	4,25	2,66
5	3,76	0,02	0,76	3,50	3,24	3,20
6	3,73	-0,06	0,69	3,42	3,32	2,92
7	3,92	0,48	0,62	3,67	3,52	3,30
8	4,04	0,68	0,64	3,64	3,10	3,15
9	2,16	0,01	0,45	2,50	2,04	1,73
10	3,40	-0,01	0,83	3,10	3,20	2,12
11	3,44	-0,05	0,58	3,25	2,93	2,52
12	4,00	-0,11	0,76	3,52	4,23	2,10
13	3,41	-0,11	0,55	3,46	2,93	2,85
14	3,81	-0,03	0,70	3,67	3,55	2,93
15	3,77	-0,02	1,15	3,32	4,03	0,91
16	3,49	0,23	0,59	3,73	2,89	3,49
17	3,59	0,07	0,59	3,34	2,99	2,67
18	3,26	-0,05	0,64	2,97	3,47	1,47
19	3,51	0,00	1,00	3,29	3,75	2,12
20	3,36	-0,05	0,56	3,32	3,08	2,72
21	3,77	-0,09	0,55	3,45	3,48	2,72
22	3,80	0,05	0,61	3,72	3,39	3,15
23	3,62	0,01	0,67	3,72	3,24	2,88
24	3,12	0,06	0,51	3,59	2,47	2,96
25	0,78	2,04	0,52	2,08	0,58	2,04
26	5,60	-0,17	5,10	0,00	4,65	0,00
27	2,68	0,02	0,52	2,98	1,63	1,99
28	2,90	0,58	0,61	2,99	2,20	2,45
29	3,65	0,01	0,65	3,49	2,29	3,27
30	3,75	-0,06	0,60	3,57	3,41	2,69
k	0,82	0,07	0,22	0,65	0,60	0,42

Table 7

Influence of DO concentration on fluorescent signal for 2 hours hybridisation.

DO concentration [pM]	PEG 6000	
	w/o	15%
200	3,09	3,35
63,2	3,01	3,38
20	2,91	3,56
6,3	2,72	3,47
2	2,53	3,5

Claims

1. A method for the detection of the allelic state of n single nucleotide polymorphism (SNP)-loci on genomic DNA comprising the steps of

(a) contacting said genomic DNA containing SNP-loci with n detector oligonucleotide sets (DO-sets), wherein n represents the number of specific loci to be tested, and wherein each DO-set represents a specific collection of
(aa) a first detector oligonucleotide (DO),
wherein said first DO comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination and a Region III, for locus specific hybridization, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and
(ab) a second DO,
wherein said second DO comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and
(ac) a third DO,

wherein said third DO is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridization, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ teminus to the 5′ terminus of Region V; and

wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets;

(b) carrying out a cyclic ligation reaction simultaneously with n DO-sets, wherein for each particular DO set of step (a) depending on the allelic state of the corresponding locus, either the first DO or the second DO or both DOs are linked to the third DO;

(c) carrying out a first PCR-amplification reaction on the ligated products with a common first and a common second PCR primer, wherein said common first PCR primer corresponds in sequence to Region I/I′ of the first and second detector oligonucleotide of step (a) and said common second PCR primer is complementary in sequence to Region V of the third DO of step (a);

(d) separating the amplification products into n aliquots;

(e) carrying out for each aliquot

(ea) a second PCR-amplification reaction with the common first PCR primer of step (c) and a second PCR primer which is either complementary to Region IV

or, optionally, to Region VI of the third DO of step (a), simultaneously or step-by-step with

(eb) an allele discrimination reaction which detects Regions II and II′ in the amplification products.

2. The method of claim 1, wherein only one SNP locus is tested and wherein the first PCR amplification reaction of step 1(c) is performed simultaneously or step by step with the allele discrimination reaction of step 1(eb) and wherein steps 1(d) and 1(ea) are omitted.

3. The method of claims 1 or 2, wherein a first oligonucleotide labelled with a first detectable label and able to hybridize with said Region II, and a second oligonucleotide labelled with a second detectable label and able to hybridize with said Region II′ are used in the allele discrimination reaction of step 1 (eb).

4. The method of claim 3 wherein said first and second detectable labels are two distinguishable fluorescent labels and their fluorescence is suppressed by quenchers located in close vicinity to said fluorescent labels and wherein the presence of Region II in the amplification products in the allele discrimination reaction of step 1 (eb) induces activation of the first fluorescent label but does not activate the second fluorescent label and the presence of Region II′ in the amplification products in the allele discrimination reaction of step 1 (eb) induces activation of the second fluorescent label, but does not activate the first fluorescent label.

5. The method of any one of claims 1 to 4, wherein both said quencher and said first fluorescent label are covalently attached to the first oligonucleotide and wherein both said quencher and said second fluorescent label are covalently attached to the second oligonucleotide and spacial separation is performed by Taq polymerase 5′-exonuclease digestion of the first oligonucleotide hybridized to Region II or the second oligonucleotide hybridized to Region II′.

6. The method of any one of claims 1 to 4, wherein both said quencher and said first fluorescent label are covalently attached to the first oligonucleotide and

wherein both said quencher and said second fluorescent label are covalently attached to the second oligonucleotide and each of the first and second oligonucleotide contains two short complementary regions and wherein said first or second fluorescent label and said quencher are brought together when said complementary regions are hybridised with each other, and wherein the separation of said first or second fluorescent label from the quencher is due to change of spacial conformation of the first or second labelled oligonucleotide after hybridization with Region II or II′.

7. The method of any one of claims 1 to 4, wherein said first and second oligonucleotides contain covalently attached fluorescent labels and said quenchers are attached to additional oligonucleotides, wherein said additional oligonucleotides are complementary to said first and second oligonucleotides and wherein said first or second fluorescent label and said quencher are brought together when said additional oligonucleotides are hybridised with said first and second oligonucleotides and wherein said quencher is separated from said fluorescent label when said first and second oligonucleotides hybridize with Regions II or II′.

8. The method of any one of claims 1 to 3, wherein the allele discrimination reaction of step 1(eb) is performed with microarray detection system simultaneously or step by step with the first amplification reaction of step 1(c) wherein individual elements of said microarray are able to hybridize with locus-specific Regions IV or, optionally, Regions VI of amplification products and wherein Regions II and II' in the amplification products are detected by hybridization with said first and second oligonucleotides labeled with two distinguishable fluorescent labels.

9. The method of any one of claims 1 to 7, wherein said common second PCR primer is destroyed after the first PCR-amplification of step 1(c).

10. The method of claim 9, wherein said common second PCR primer contains Uridine- or Ribo-nucleotides and is destroyed by Uracile DNA Glycosylase

(UDG) or RNase included in said second PCR-amplification reaction of step 1(e).

11. The method of any one of claims 1 to 10, wherein said Regions I and I′ have identical sequences.

12. The method of any one of claims 1 to 11, wherein said first, second and third DOs are prepared by ligation based synthesis.

13. The method of any one of claims 1 to 12, wherein the concentration of DOs in each DO set is in the range of 10pM to 10nM, preferably 1nM.

14. The method of any one of claims 1 to 13, wherein only one cycle of ligation reaction is performed in step 1(b), preferably more than 10 cycles, and most preferably 15 to 25 cycles.

15. The method of any one of claims 1 to 14, wherein the cyclic ligation reaction of step 1(b) is effected with thermostable DNA ligase, preferably Taq DNA ligase, Tth DNA ligase, and most preferably with Pfu DNA ligase.

16. The method of any one of claims 1 to 15, wherein the cyclic ligation of step 1(b) is effected in the presence of at least 1% polyethyleneglycol (PEG), preferably in the presence of at least 5% PEG and most preferably in the presence of about 15% PEG.

17. The method of claim 16, wherein said polyethylene glycol has a molecular weight of more than 200, preferably more than 3000 and most preferably in the range 6000 – 8000.

18. The method of any one of claims 1 to 17, wherein the cyclic ligation of step 1(b) is effected with at least 2 subcycles, preferably with 3 to 6 subcycles.

19. A detector oligonucleotide (DO) selected from the group consisting of

(a) a first DO which comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination and a Region III, for locus specific hybridization, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and

(b) a second DO, which comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and

(c) a third DO, which is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridization, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ teminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is

invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets;

20. The detector oligonucleotide of claim 19, wherein said detector oligonucleotide comprises a nucleotide sequence as set forth in SEQ ID NOs 1 to 129.

21. Use of the detector oligonucleotide of claims 19 or 20 for allele discrimination of biallelic blocks of closely located nucleotide substitutions or small insertions/deletions, wherein Region III comprises a DNA sequence which corresponds to the first allelic state of said block and Region III′ comprises a DNA sequence which corresponds to the second allelic state of said block and wherein Region III and III′ have different 3′ terminal nucleotides.

22. Kit comprising one or several detector oligonucleotide sets, wherein each set comprises a first, a second and a third detector oligonucleotide (DO), wherein said first DO comprises, in 5′ to 3′ orientation, a Region I comprising a DNA sequence for PCR amplification; a Region II, or alternatively, II′ for allele discrimination and a Region III, for locus specific hybridization, wherein said Region III comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the first allelic state or, alternatively, for the second allelic state of said SNP-locus; and
wherein said second DO comprises, in 5′ to 3′ orientation, a Region I′ corresponding in sequence to Region I of the first DO; a Region II′, or alternatively, Region II for allele discrimination, wherein, when Region II is comprised in the first DO, the second DO must comprise Region II′ or, alternatively, when Region II′ is comprised in the first DO, the second DO must comprise Region II; and a Region III′, wherein said Region III′ comprises a DNA sequence which corresponds to the part of the sequence of the SNP locus located upstream of the polymorphic single nucleotide site and which consists, at its 3′ terminus, of a single nucleotide complementary to the single nucleotide specific for either the second allelic state or, alternatively, for the

first allelic state of said SNP-locus, wherein, when said Region III is specific for said first allelic state, said Region III′ is specific for said second allelic state or, alternatively, when said Region III is specific for said second allelic state, said Region III′ is specific for said first allelic state of said SNP-locus; and wherein said third DO is 5′-phosphorylated and comprises, in 5′ to 3′ orientation, a Region IV for locus specific hybridization, wherein said Region IV comprises a DNA sequence which corresponds in sequence to the part of the sequence of the SNP locus located downstream of the polymorphic single nucleotide site and which, at its 5′ terminus is located immediately downstream of the 3′ terminus of the first or second DO; and a Region V comprising a DNA sequence for PCR amplification; and, optionally, a Region VI for locus discrimination, wherein said Region VI comprises a DNA sequence specific for each DO-set and which is linked at its 5′ terminus to the 3′ terminus of Region IV and at its 3′ teminus to the 5′ terminus of Region V; and wherein said Regions I/I′ and II/II′ are invariable in sequence in all said first and second detector oligonucleotides in all DO-sets and said Region V is invariable in sequence in all said third detector oligonucleotides in all DO-sets, and wherein said Region VI is not identical in sequence in the different DO-sets.

FIGURES

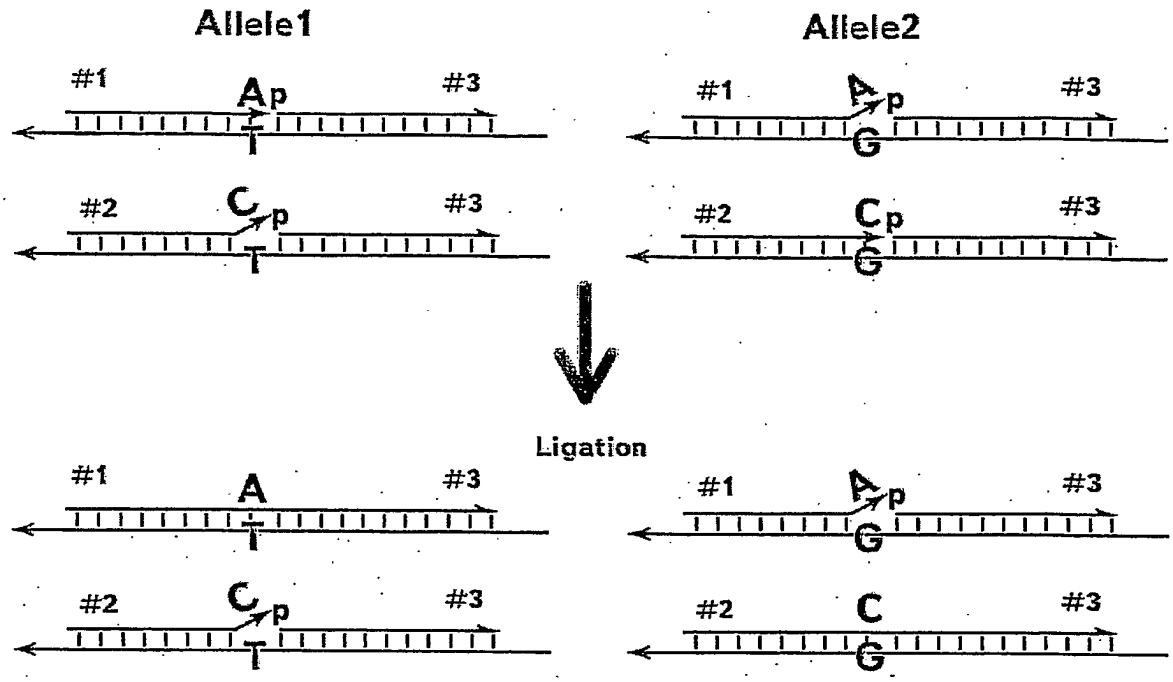


Figure 1

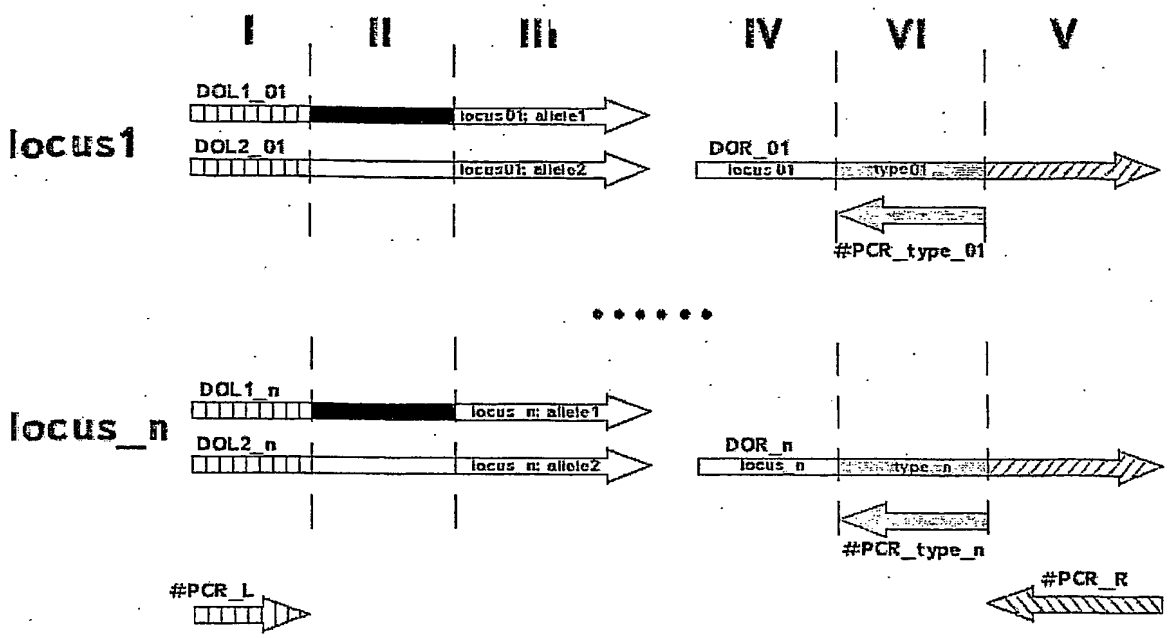


Figure 2.

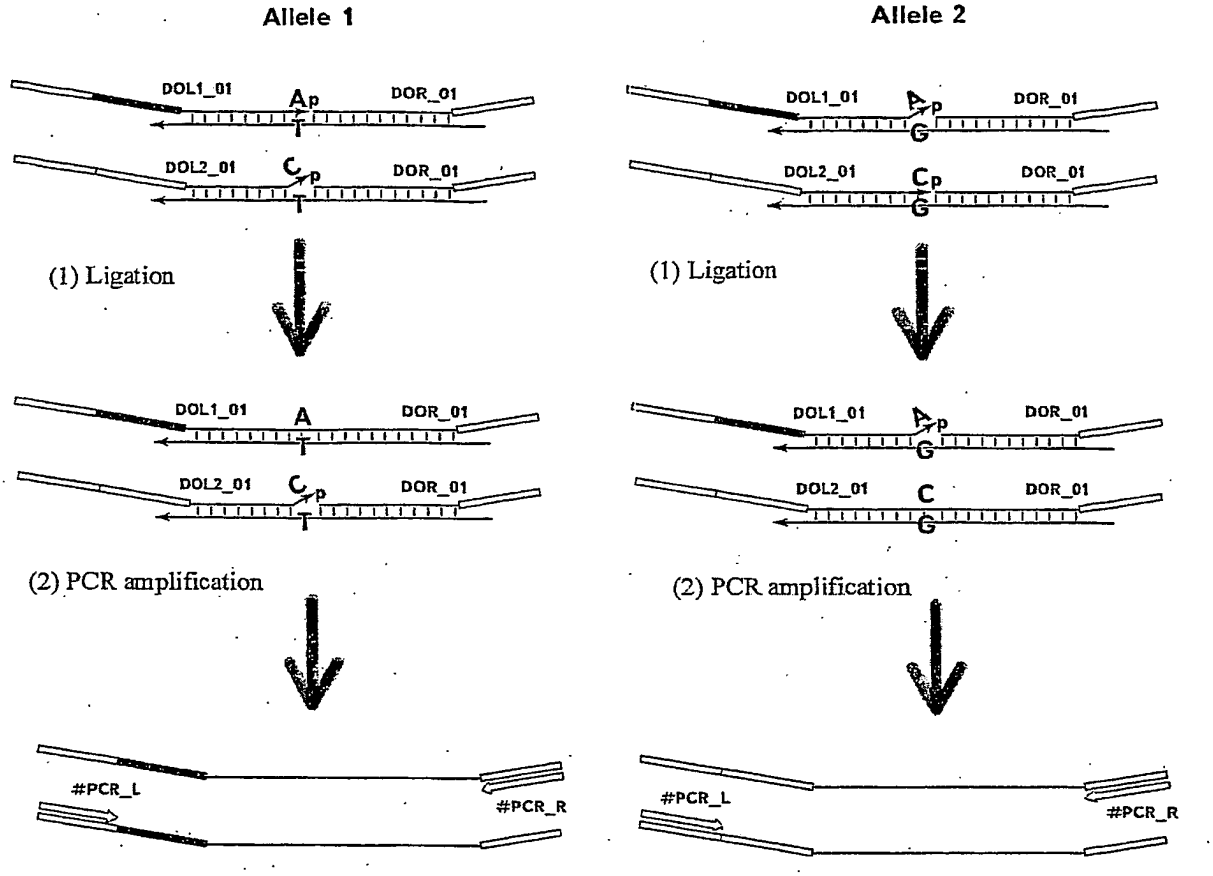

Allele 1
Allele 2
DOL1_01
DOR_01
DOL2_01
(1) Ligation
(2) PCR amplification
#PCR_L
#PCR_R

Figure 3.

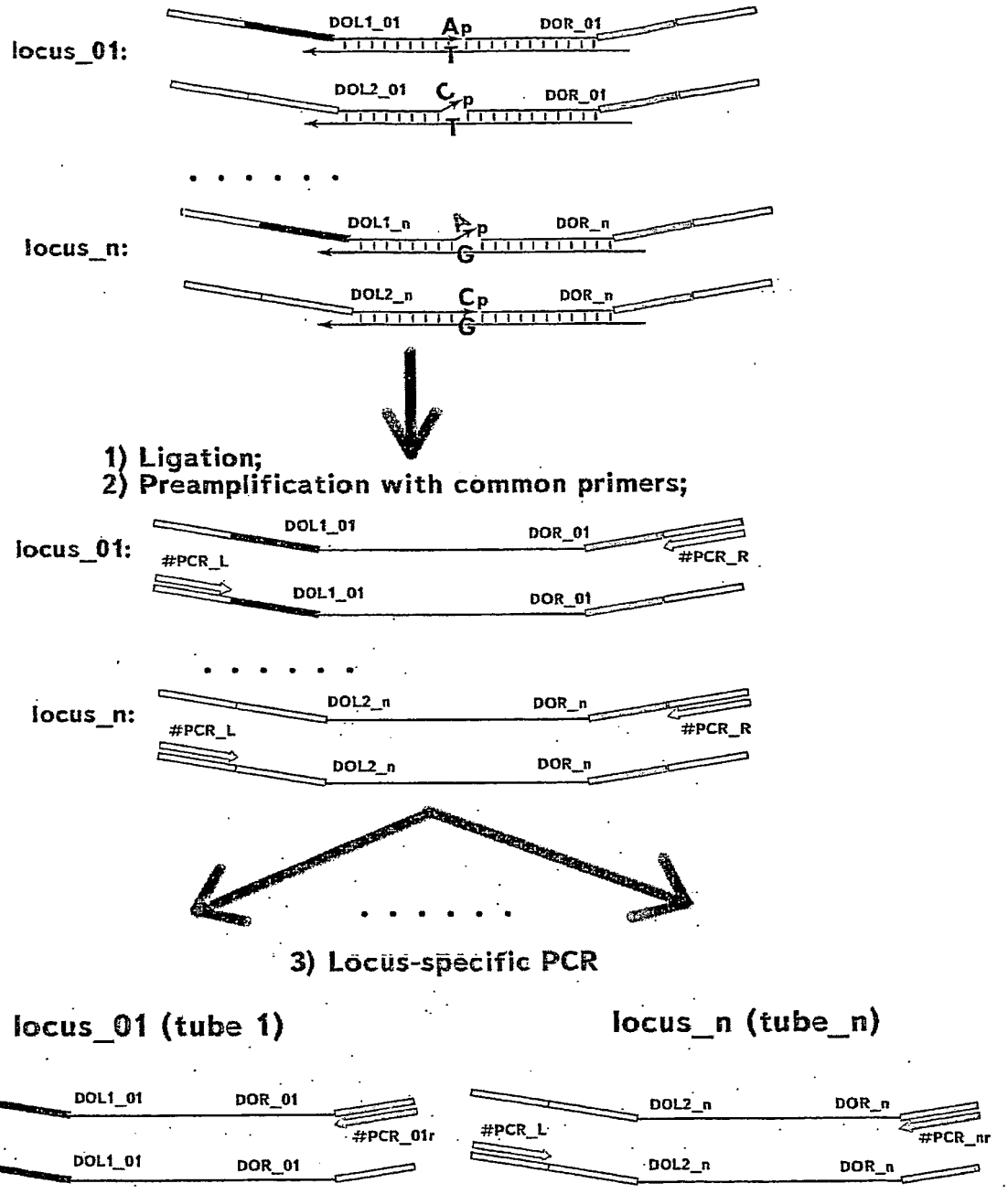


Figure 4.

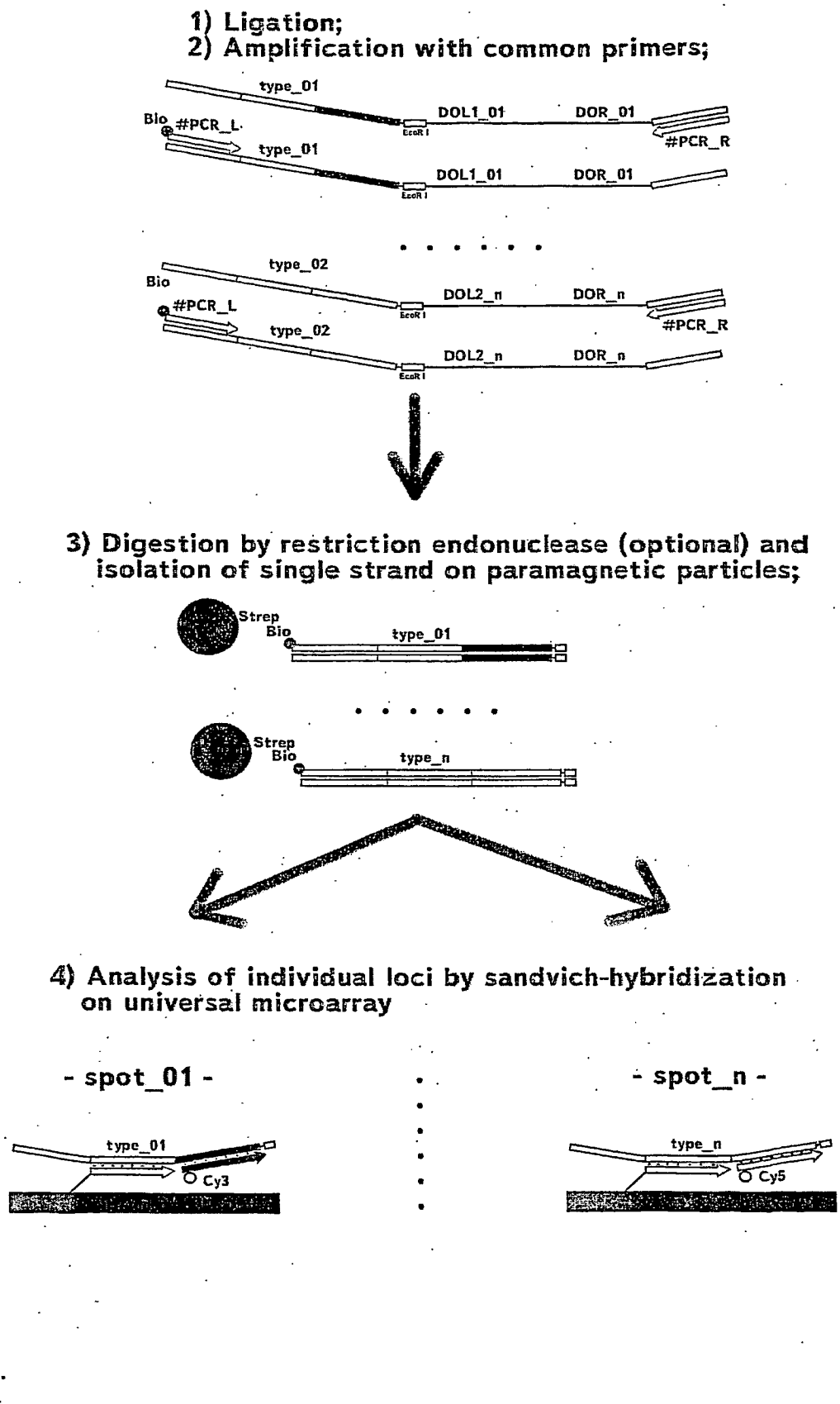
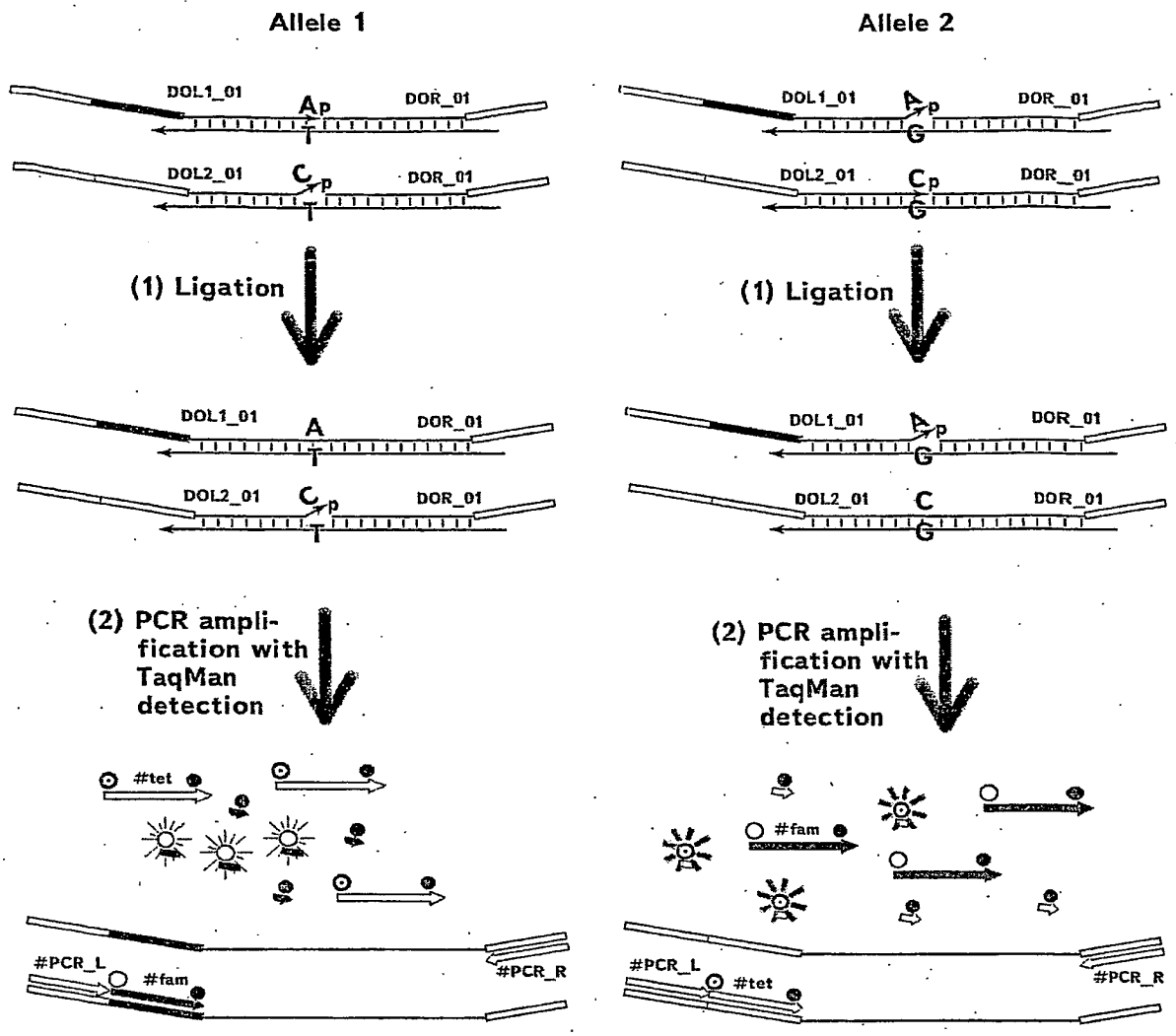

1) Ligation;
2) Amplification with common primers;
type_01
Bio
#PCR_L
DOL1_01
DOR_01
EcoR I
#PCR_R
type_01
DOL1_01
DOR_01
type_02
Bio
#PCR_L
DOL2_n
DOR_n
EcoR I
#PCR_R
type_02
DOL2_n
DOR_n
3) Digestion by restriction endonuclease (optional) and isolation of single strand on paramagnetic particles;
Strep
Bio
type_01
Strep
Bio
type_n
4) Analysis of individual loci by sandvich-hybridization on universal microarray
- spot_01 -
- spot_n -
type_01
Cy3
type_n
Cy5

Figure 5.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Allele 2
DOL1_01
DOR_01
DOL2_01
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(2) PCR amplification with TaqMan detection
#tet
#fam
#PCR_L
#PCR_R

Figure 6.

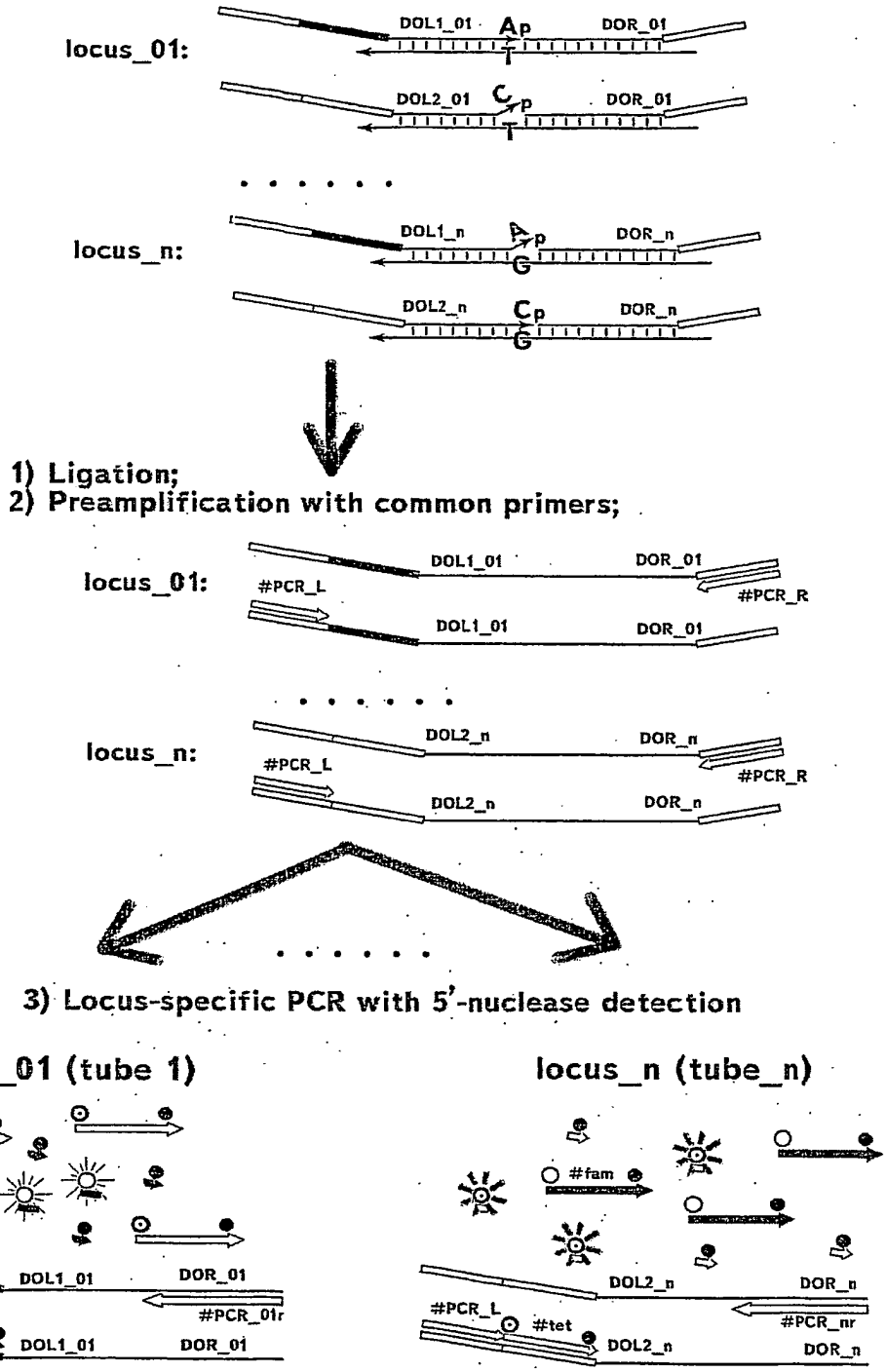
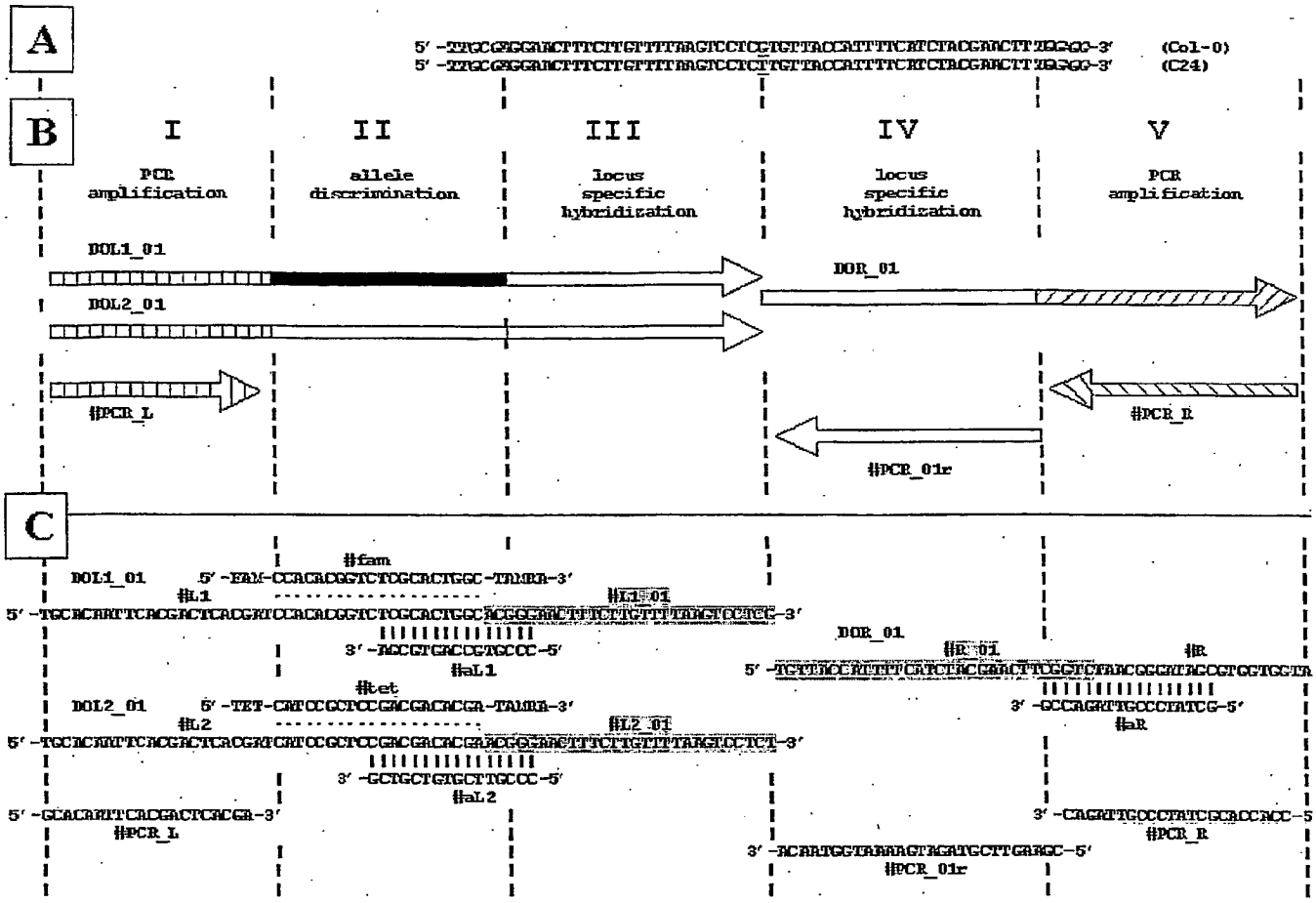

locus_01:
DOL1_01
Ap
DOR_01
DOL2_01
Cp
DOR_01
locus_n:
DOL1_n
Ap
DOR_n
DOL2_n
Cp
DOR_n
1) Ligation;
2) Preamplification with common primers;
locus_01:
DOL1_01
DOR_01
#PCR_L
#PCR_R
DOL1_01
DOR_01
locus_n:
DOL2_n
DOR_n
#PCR_L
#PCR_R
DOL2_n
DOR_n
3) Locus-specific PCR with 5'-nuclease detection
locus_01 (tube 1)
#tet
DOL1_01
DOR_01
#PCR_L
#fam
#PCR_01r
DOL1_01
DOR_01
locus_n (tube_n)
#fam
DOL2_n
DOR_n
#PCR_L
#tet
#PCR_nr
DOL2_n
DOR_n

Figure 7.


A
B
C
I
II
III
IV
V
PCR amplification
allele discrimination
locus specific hybridization
locus specific hybridization
PCR amplification
DOL1_01
DOL2_01
DOR_01
#PCR_L
#PCR_R
#PCR_01r
(Col-0)
(C24)
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#tet
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#aL1
#aL2
#R_01
#aR

Figure 8.

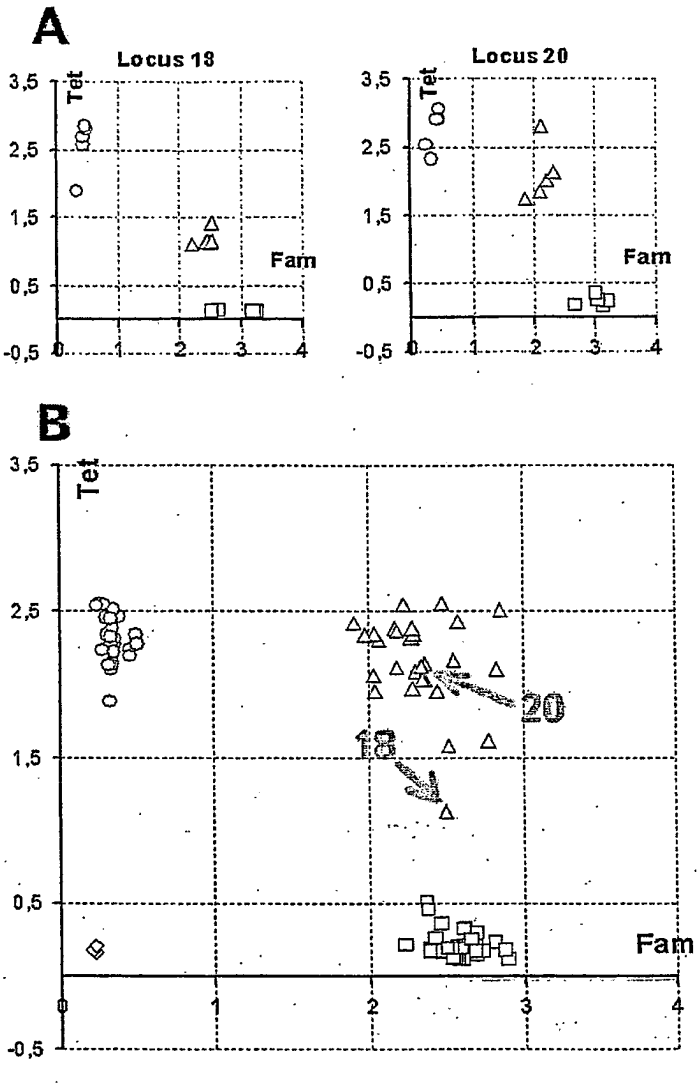


Figure 9.

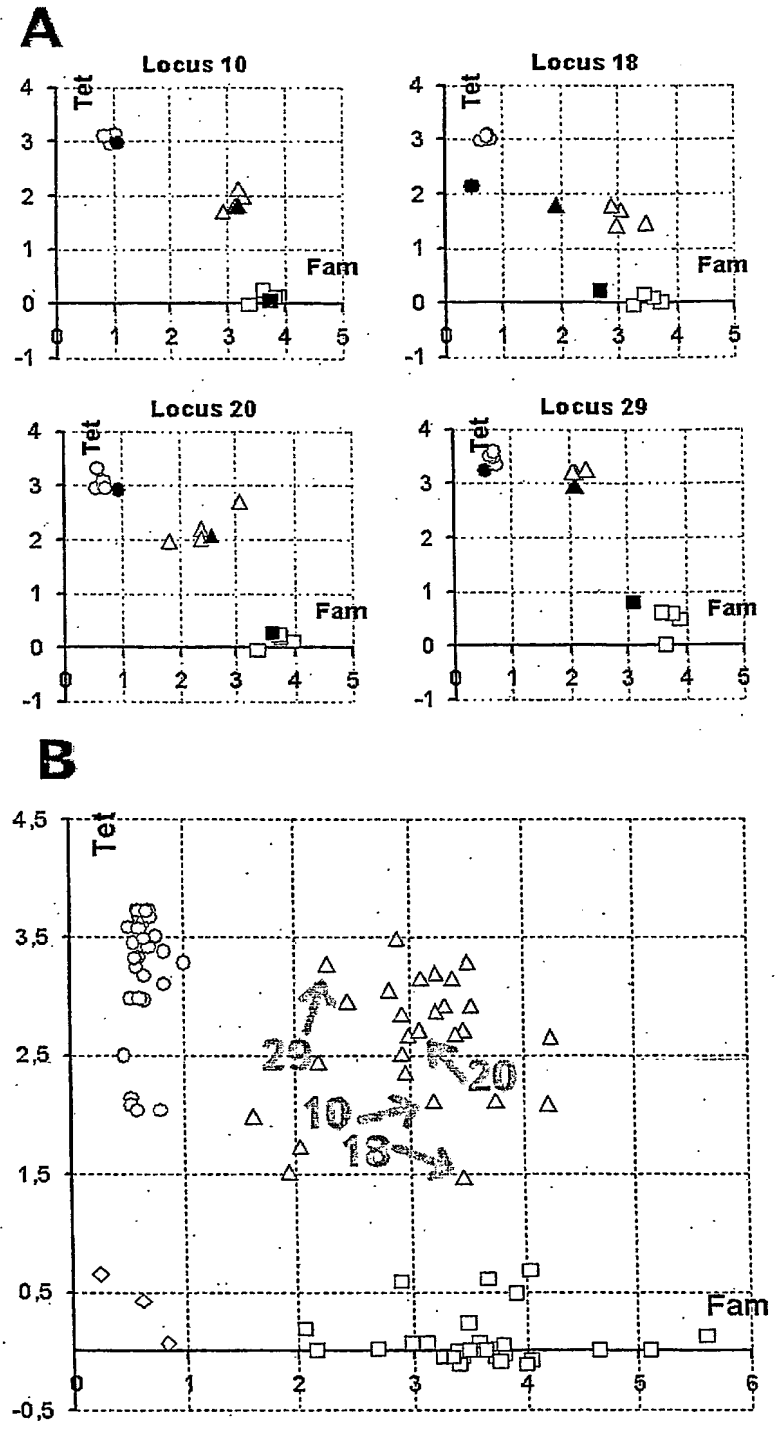
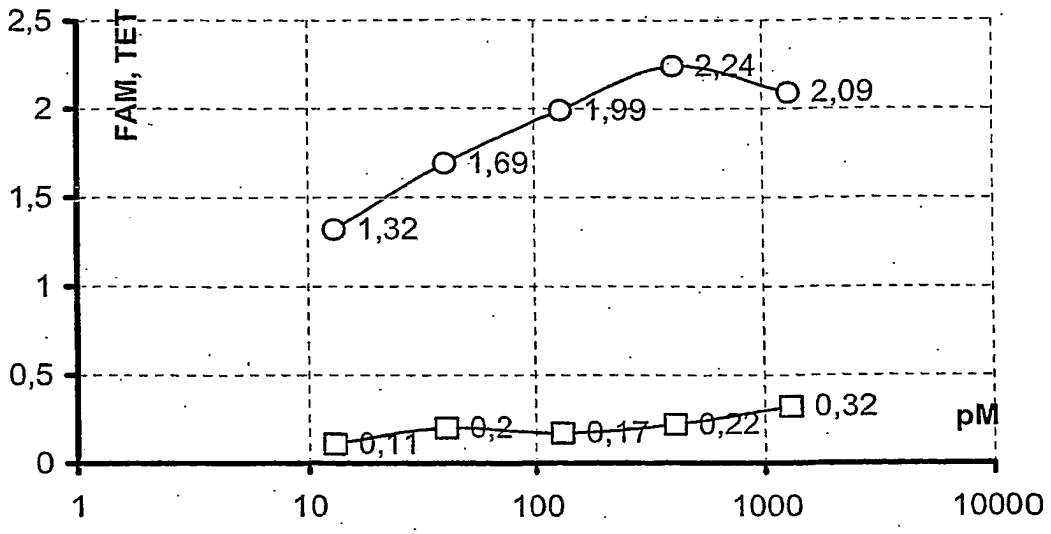

A
Locus 10
Locus 18
Locus 20
Locus 29
Tet
Fam
B
Tet
Fam
10
18
20
29

Figure 10.


2,5
2
1,5
1
0,5
0
FAM, TET
1,32
1,69
1,99
2,24
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0,11
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0,17
0,22
0,32
pM
1
10
100
1000
10000

Figure 11.

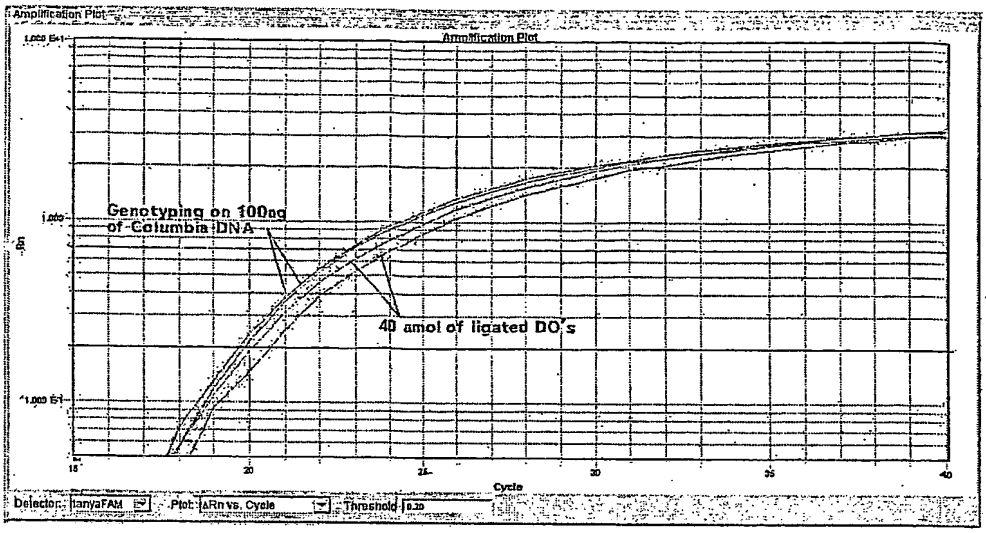

Figure 12

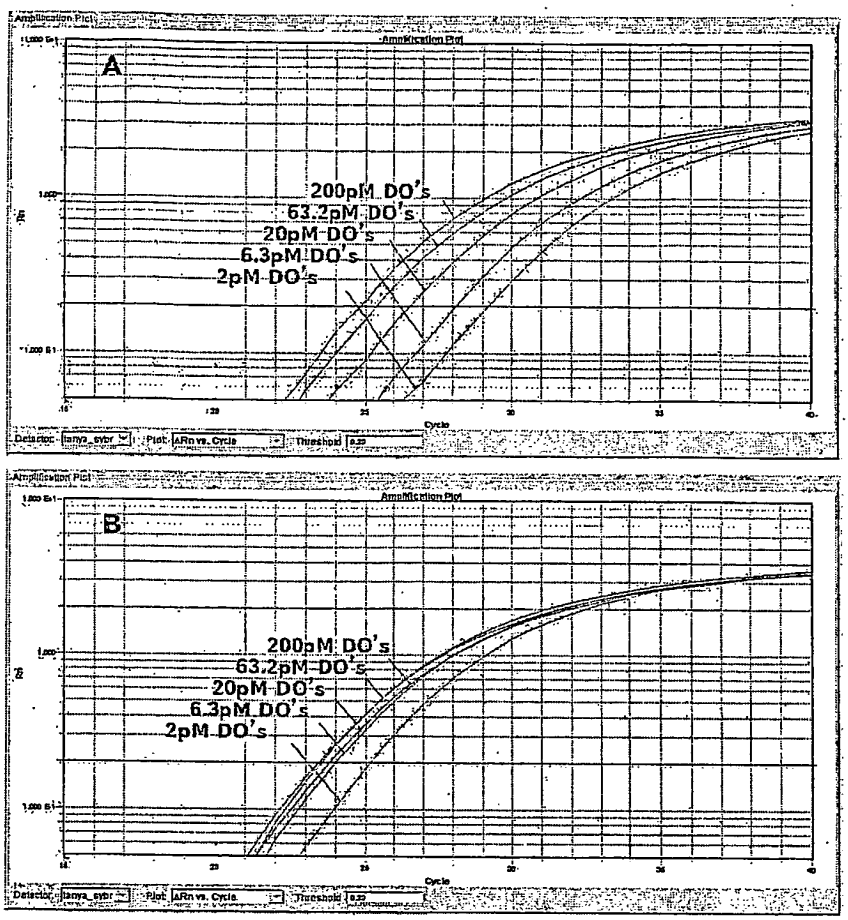
A
200pM DO's
63.2pM DO's
20pM DO's
6.3pM DO's
2pM DO's
Amplification Plot
Cycle
B
200pM DO's
63.2pM DO's
20pM DO's
6.3pM DO's
2pM DO's
Amplification Plot
Cycle

Figure 13

SEQUENCE LISTING

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<120> Ligation-based method of analysis of single nucleotide polymorphisms on genomic DNA

<130> H1721 PCT

<160> 129

<170> PatentIn version 3.1

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<220>

<223> Oligonucleotide

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<211> 29

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<213> Artificial Sequence

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